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Determination of the influence of factors (ethanol, pH and a_w) on the preservation of cosmetics using experimental design

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Synopsis

OBJECTIVE: Ethanol, pH and water activity are three well-known parameters that can influence the preservation of cosmetic products. With the new constraints regarding the antimicrobial effectiveness and the restrictive use of preservatives, a D-optimal design was set up to evaluate the influence of these three parameters on the microbiological conservation.

METHODS: To monitor the effectiveness of the different combination of these set parameters, a challenge test in compliance with the International standard ISO 11930: 2012 was implemented. The formulations established in our study could support wide variations of ethanol concentration, pH values and glycerin concentration without noticeable effects on the stability of the products.

RESULTS: In the conditions of the study, determining the value of a single parameter, with the tested concentration, could not guarantee microbiological conservation. However, a high concentration of ethanol associated with an extreme pH could inhibit bacteria growth from the first day (D0). Besides, it appears that despite an a_w above 0.6 (even 0.8) and without any preservatives incorporated in formulas, it was possible to guarantee the microbiological stability of the cosmetic product when maintaining the right combination of the selected parameters.

CONCLUSION: Following the analysis of the different values obtained during the experimentation, there seems to be a correlation between the a_w and the selected parameters aforementioned. An application of this relationship could be to define the a_w of cosmetic products by using the formula, thus avoiding the evaluation of this parameter with a measuring device.

Résumé

OBJECTIFS: L'alcool, le pH et l'activité de l'eau sont les trois paramètres bien définis qui peuvent influencer la conservation des produits cosmétiques. Avec les nouvelles contraintes concernant l'efficacité antimicrobienne et la limite d'utilisation de conservateurs, un plan D-optimal est mis en place pour évaluer l'influence de ces trois paramètres sur la conservation microbiologique.

METHODES: Afin de vérifier l'efficacité des différentes combinaisons de ces paramètres, un challenge test est réalisé selon la norme internationale ISO 11930:2012. Les formulations utilisées dans notre étude ont permis de tester de grandes variations d'alcool, de pH et de glycérine, sans effets notables sur la stabilité des produits.

RESULTATS: Dans les conditions de l'étude, l'évaluation de la valeur d'un seul paramètre, aux concentrations testées, ne permet pas de garantir une conservation microbiologique satisfaisante. Cependant, une forte concentration d'éthanol associée à un pH extrême permet d'inhiber la croissance des bactéries dès la première journée (D0) du challenge test. De plus, il apparaît que malgré une a_w supérieure à 0,6 (voire 0,8) et en l'absence de conservateurs dans les formules, il est possible de garantir la stabilité microbiologique du produit cosmétique lorsque l'on maîtrise les bonnes combinaisons des paramètres définis au préalable.

CONCLUSION: L'analyse des résultats a permis de mettre en avant une corrélation entre l'a_w et les paramètres cités en amont. L'utilisation de cette équation permettrait d'appréhender la valeur de l'a_w d'un produit cosmétique à partir de sa formule et cela éviterait alors une évaluation de ce paramètre avec un appareil de mesure.

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Introduction

Today, the preservation of cosmetics products is an important challenge [1–4]. Indeed, cosmetic companies have more and more constraints: preservatives are often questioned and regulations require the microbiological safety of such products. For instance, in the new Regulation (EC) No 1223/2009 on cosmetic products, it is required that the results of the challenge test (CT) for cosmetic products be provided [5–7].

Preserving cosmetic is mandatory for two main reasons. The first concerns health hazards. When microorganisms contaminate a cosmetic product, this can be harmful to consumers (e.g. skin infections). The second reason is product spoilage. Microorganisms' proliferation can cause stability issues, pH, colour or odour changes [8].

Given the composition of most cosmetic products, these products are considered as good growth media for microorganisms. Therefore, without an effective conservation system, the development of several bacteria, mold or yeast is highly likely. This is contrary to the expectations of the ANSM («manufacturers guarantee that their products comply with the legislative, regulatory, present no health hazard») [9] and to those of the customers.

The aim of the study was to determine when the CT could be avoided and guaranteeing that the microbiological risk is con-

trolled, as required by the International standard ISO 11930: 2012 [10]. To reach this goal, an efficient conservation system is defined without adding any preservative, i.e. combinations of the three following parameters are tested: pH, water activity (a_w) and ethanol concentration within the final product. The variation of glycerin concentration allows to modify the a_w values. Indeed, these three parameters could influence the microbial growth in these products [11–15].

The experimental design approach is often used for determining the most optimal component combinations in the drug, food or cosmetic industries [16]. Here, a D-optimal design is used for programming the experiments schedule. This method provides information feedback on the influence of the three factors on the microbiological growth of the selected microorganisms (using a fewer number of experiments). Furthermore, it also allows us to approximate the factors associated with the 'most' effective systems. To evaluate the microbiological risk of each preparation, a CT is established in compliance with the International standard ISO 11930: 2012 1 antimicrobial preservative efficacy test [10]. The performance of the different combination of the three parameters to inhibit the growth of microorganisms is evaluated by the statistical analyses of the CT results.

Materials and methods

Raw materials

Sixteen products were formulated by emulsion O/W with the same matrix.

The topical preparations used in this work comprised three phases:

- a hydrophilic phase (called Phase 1 in Table I) containing demineralized water, glycerin and Surfhope. This phase also contains the following rheology modifiers: Corn starch, Natrosol and Rhodicare T. Their concentrations rise at the same time as those of ethanol.
- a lipophilic phase (called Phase 2 in Table I) containing Emulium Kappa, Cetiol OE and Sunflower oil
- a phase called 3, in Table I, containing ethanol

Each raw material was of cosmetic grade and was sampled by suppliers (Table I).

Ethanol and glycerin ranged from 0% to 20% and pH from 4 to 11. The a_w was a parameter measured and not imposed. A_w ranged from 0.8 to 0.96.

The optimized formulas compositions (Ingredients, INCI name, Supplier and quantities) are summarized in Table I.

Formulation

The two phases (L & H) were heated separately at a 75°C temperature, and the lipophilic phase (L) was then incorporated into the hydrophilic phase (H) by stirring at 2000 rpm for 15 min. This step was followed by stirring at 1500 rpm until the temperature reached a maximum of 35°C. Once the temperature reached 30°C, the ethanol (phase A) was added.

Analysis

When the emulsion was at room temperature, the pH was adjusted with a pH-meter and the a_w was measured with a Novasina LabMaster-Aw®. The stability was also evaluated with a centrifuge (Sigma, Fisher Bioblock Scientific®, St Louis, MO, U.S.A) at 2594 **g** for 20 min.

Experimental design

The software Design Expert® is a Design of Experiments (DOE) software. It offers to plan, estimate and control the statistics and models for factorial and no-factorial designs. It also draws controlling plots for the incoming data. It can handle many models of experimental design such as standard factorial design, response surface or mixture design. In this paper, the surface response design based on the D-optimal criterion was selected to evaluate and to estimate the effects of combined preservatives on antimicrobial efficacy. Such a design provides maximum information from a limited number of experiments [16]. Moreover, when all the results are expressed as models, it is possible to determine mathematically or graphically, a composition satisfying the required criteria [16].

The aim of the present study was to assess the influence of the factors on the viability of the growth of the microorganisms. To do so, we measured the effect of varying the values of the factors within a specific range.

Table I Final formula

Phases	Ingredients Commercial Name	INCI name	Supplier	Quantities (%)
Phase 1	/	Demineralized Water	EBI	QSP
	/	Glycerin	Cooper	0–20
	Surfhope	Sucrose palmitate	Mitsubishi-Kagaku	1
	Corn starch	Zea mays (corn) starch	National	2–3
	Natrosol	Hydroxyethylcellulose	Hercules	2–3
	Rhodicare T	Xanthan gum	Rhodia	0.5–0.7
Phase 2	Emulium Kappa	Candelilla/Jojoba/Rice Bran Polyglyceryl-3 Esters (and) Glyceryl Stearate (and) Cetearyl Ethanol (and) Sodium Stearoyl Lactylate	Gattefossé	2.5
	Cetiol OE	Dicaprylyl ether	Cognis	4
	Sunflower oil	Sunflower oil	Cooper	4
Phase 3	/	Ethanol	Fisher Chemical	0–20

Definition of the constraints and the factors tested

Three factors were investigated: pH, alcohol and Aw (based on the Glycerin concentration)

- pH: The pH optimum for growth of most microorganisms is around neutrality. Extreme pH, acidic or basic pH can completely inhibit microbial growth. The pH range where growth is possible depends on the microorganisms (Table II) [10, 17]. A pH meter GLP21, Crison® (Barcelona, Espagne) was used at the end of the formulation. The emulsion was basified with sodium hydroxide and acidified with citric acid.
- Ethanol: The minimal ethanol concentration to inhibit microbial growth depends on the type of microorganisms (Table II) [10, 17]. The ethanol used was absolute ethanol (Fisher Chemical) at 99.9%.
- a_w: The microorganisms require the presence of bioavailable water for their metabolism and growth [10, 17]. The proportions of a water free product, aw (Table III) was measured by an Aw-meter Labmaster-Aw, Novasina® (Lachen, Switzerland). Glycerin was the factor impacting the a_w.

Several formulations were prepared from the basic formulation by changing these three factors (pH, ethanol et a_w). There is a wide variety of formulas in the product market, the levels of the selected factors are suited with extreme formulations, e.g. epilation products, peeling products, alcoholic products, etc.

The levels are (called the codes of box for the values −1/+1):

- pH from 4 to 11
- Ethanol concentration from 4% (v/v) to 20% (v/v)
- Glycerin concentration from 4% (v/v) to 20% (v/v)

Sixteen runs were generated using the surface response design based on the D-optimal criterion for quadratic models. This criteria is commonly used for small samples (). To summarize, the following method was used: the Analysis of Variance (ANOVA) for a list of usual polynomial models was implemented. When the ANOVA test of one of the model was significant, the latter was considered as a 'good' candidate model to be the final model. Next, the less complex model issued from all the candidate model was selected based on the principle of parsimony. Eventually, the consistency of the error distribution (qq plots, plots of standardized residuals) with the hypothesis of the ANOVA was checked. In addition, two formulas were performed in duplicate: formulas 2–8 and formulas 4–15. Experiments 16 were conducted without alcohol with pH 7.5 and a_W 0.96, this last mixture was chosen to assess the predictive value of the mathematical model. (Table III).

We could not implement all the measures for the 16 testing formulas. Therefore, the measures were implemented on 2 days: 'Day_0' was dedicated to formulas 1–8 and 'Day_1' to formulas 9–16. Therefore, the blocking factor (linked to this experimental effect) could be estimated. It was thus verified that this blocking factor was not significant. The list of the 16 runs is presented in Table III.

Table II Ranges values allowing micro-organisms growth [10, 17]

	Ranges of optimum pH	Minimal ethanol concentration (w/w)	Minimum a_w value
Bacteria	4–9	8–11% in volume	*Pseudomonas aeruginosa*: 0.97 *Escherichia coli*: 0.95 *Staphylococcus aureus*: 0.86
Yeast	1.5–8	8–11% in volume	*Candida albicans*: 0.70
Mold	5–11	15–18% in volume	*Aspergillus brasiliensis*: 0.77

Antimicrobial preservative efficacy test in compliance with the French standard AFNOR NF T75-611

Pseudomonas aeruginosa, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Aspergillus brasiliensis* constitute a panel of germs issued from different biotopes such as water, skin, intestinal and environmental, and which could contaminate cosmetics. They also represent potentially pathogenic germs. Indeed, a contamination by these germs could cause illnesses ranging from simple skin infections to respiratory diseases or anaphylactic shock. The efficacy of the preservatives against various microorganisms was measured using the method recommended by the International standard ISO 11930: 2012 [10].

Formulations were tested for prevention against the following microorganisms: *Pseudomonas aeruginosa* ATCC 9027, CIP 82.118, *Staphylococcus aureus* ATCC 6538, CIP 4.83, *Escherichia coli* ATCC 8739, CIP 53.126, *Candida albicans* ATCC 10231, IP 48.72, *Aspergillus brasiliensis* ATCC 16404, IP 1431.83.

The three bacterial strains were inoculated separately at a concentration of 1×10^6 CFU mL^{-1}, 1×10^5 CFU mL^{-1} for yeast and 1×10^4 CFU mL^{-1} for mold. Three dilutions for each microorganisms and each formula were performed during the neutralization. Samples were incubated at room temperature (22.5 ± 2.5°C), and the aerobic plate counts were measured, as required by the French standard AFNOR NF T75-611, on the first day (D0), 7th day (D7), 14th day (D14) and 28th day (D28). The log reduction (LR) values for the bacterial and fungal counts were calculated as follows:

$$Rx = \log(Ni) - \log(Nx)$$

Ni: number of microorganism inoculated (determined with the measures at 48 h)

Nx: number of survivors at each sampling time [10]

Table III Design Expert® experimental fields

Run	Block	Ethanol concentration (%)	pH	Glycerin concentration (%)	a_w
1	Day_0	12	7.5	8	0.88
2	Day_0	20	4	4	0.87
3	Day_0	12	7.5	20	0.84
4	Day_0	4	4	4	0.94
5	Day_0	20	11	4	0.86
6	Day_0	4	11	4	0.93
7	Day_0	20	11	20	0.80
8	Day_0	20	4	4	0.87
9	Day_1	20	4	20	0.81
10	Day_1	20	11	12	0.84
11	Day_1	4	11	20	0.88
12	Day_1	4	4	20	0.88
13	Day_1	12	11	4	0.90
14	Day_1	20	7.5	4	0.88
15	Day_1	4	4	4	0.94
16	Day_1	0	7.5	0	0.96

Table IV The International standard ISO 11930: 2012 [10] acceptance criteria (A or B) for cosmetic products

	Logarithmic reduction			
	Criteria	D7	D14	D28
Bacteria	A	≥3	≥3 and NI	≥3 and NI
	B		≥3	≥3 and NI
Yeast	A	≥1	≥1 and NI	≥1 and NI
	B		≥1	≥1 and NI
Mold	A		≥0	≥1
	B		≥0	≥0 and NI

NI, no increase.

The Table IV shows the International standard ISO 11930: 2012 acceptance criteria (A or B) for topical application of a preparation.

Results and discussion

Challenge test

Logarithmic reduction results by species and time, expressed in log CFU mL^{-1}, were calculated from the difference between log CFU mL^{-1} at D0, D7, D14 and D28 (Table V).

The reproducibility of the runs was confirmed by the results of duplicates (formulas 2–8 and 4–15). Formula 16 (the positive control formula) with neither ethanol nor glycerin was highly contaminated from D0 to D28. Thus, without any protective parameters the contamination of formula 16 was expected.

The reduction in bacterial and fungal counts following incubation days 2, 7 and 14 at room temperature was taken as the measured response and modeled by polynomial equations (Table IV). The equations can be first, second or third order depending on various factors and their interaction: however, only the first order was significant for two species in this study (Table VI)

Table VI Significance of the results and mathematical model used at D0

Species	*P*	Significance	Mathematical model	R-Squared
E. coli	0.49	Not significant	Linear	0.42
S. aureus	0.03	Significant	Linear	0.81
P. aeruginosa	0.37	Not significant	Linear	0.73
C. albicans	0.22	Not significant	Linear	0.49
A. brasiliensis	0.03	Significant	2FI	0.76

The model of the Linear equation (no interaction between components) is:

Equation 2:

$$\mu = a_1X_1 + a_2X_2 + a_3X_3$$

where μ is the response, X_i the independent variable corresponding to the concentration of preservatives and a_i indicates the main effect of the factors

For each response an equation was obtained, the significance of the mathematical models was estimated by Analysis of Variance (ANOVA) (Table VI). This table shows the significance level, the R-squared and the selected mathematical models for each species. The names of the models are as follows: linear for the main effect model (without any factorial interaction) and 2FI for a main effect model with two-factor interactions.

R-squared statistics tells us about the correlation between the fitted (by the suited model) measures and the observed measures.

The graph 'quantile-quantile plot of residuals' was used to confirm the normality assumption. If the points were close to the

Table V Screening matrix for Logarithmic reduction by strain and mixture at D0, D7, D14 and D28

	D0					D7					D14					D28				
Formulations	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e
1	0.2	0.1	0.6	0.0	0.0	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1
2	5.8	5.8	5.8	0.2	0.2	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1
3	0.1	0.1	5.8	0.0	0.0	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1
4	0.0	0.0	0.7	0.0	0.1	5.8	5.8	5.8	1.0	0.5	5.8	5.8	5.8	2.3	1.1	5.8	5.8	5.8	2.4	5.1
5	5.8	5.8	5.8	4.8	1.1	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1
6	0.1	0.0	0.0	0.0	0.1	2.0	2.0	5.8	0.4	0.1	5.8	5.8	5.8	0.9	0.3	5.8	5.8	5.8	4.8	1.1
7	5.8	5.8	5.8	4.8	1.6	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1
8	5.8	5.8	5.8	1.2	0.1	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1
9	5.8	5.8	5.8	4.8	0.6	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1
10	5.8	5.8	5.8	4.8	0.9	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1
11	0.0	0.0	0.3	0.1	0.0	5.8	5.8	5.8	4.8	0.6	5.8	5.8	5.8	4.8	0.4	5.8	5.8	5.8	4.8	1.5
12	5.8	0.0	0.5	0.2	0.1	5.8	5.8	5.8	4.8	0.6	5.8	5.8	5.8	4.8	1.6	5.8	5.8	5.8	4.8	5.1
13	2.3	0.4	5.8	0.1	0.1	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1
14	0.5	1.4	5.8	4.8	0.2	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1	5.8	5.8	5.8	4.8	5.1
15	0.2	0.0	0.4	0.0	0.0	5.8	5.8	5.8	4.8	0.5	5.8	5.8	5.8	1.8	1.2	5.8	5.8	5.8	4.8	5.1
16	0.1	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	1.3

a: *E. coli*, b: *S. aureus*, c: *P. aeruginosa*, d: *C. albicans*, e: *A. brasiliensis*.

straight line, the standardized errors of the model could be considered to be Gaussian.

Moreover, the graph 'externally studentized residuals' was used to identify eventual model and/or data problems by highlighting outliers. Ideally, these graphics should form point clouds that are completely random.

General analysis at D0 (first day)

All the formulations containing 20% of ethanol (formulation 2, 5, 7, 8, 9, 10) showed no contamination by any of the three bacteria except formula 14. Indeed, the pH of this formula was 7.5, which greatly promoted microbial growth. Therefore, regarding bacteria growth, ethanol and pH produced a synergistic effect on the antimicrobial conservation.

Formulas containing 20% ethanol inhibited the growth of *C. albicans* except for formulas 2 and 8. In none of the formulas was inhibition of *Aspergillus brasiliensis* observed.

Analysis of results at D0 with Design Expert®

The reduction in bacterial and fungal count at D0 was selected as the measured factor. The significance of the mathematical models for each microorganism is summarized in Table VI.

The absence of microorganism growth for several of the formulas could explain why some models were not significant. For the 3 non-significant strains (*E. coli, P. aeruginosa* et *C. albicans*), there was at best a growth of 50%. This inevitably resulted in lack of contrast, thus leading to a lack of significance.

Analysis of *E. coli* at D0

Nine formulas out of sixteen had no inhibition data. Thus, proper determinations of mathematical modelization were not possible because of this missing data. Therefore, the R-squared ($R^2 = 0.42$) was low and the analysis of residuals showed a bad distribution of the points causing error prediction.

Analysis of *S. aureus* at D0

Ten formulas out of sixteen had no inhibition data. The R-squared was high ($R^2 = 0.81$). Nevertheless, data results were widely spread, and so a large block effect was demonstrated. Blocking is a restriction on the randomization of the experiment which is used to reduce error. An important block effect exhibited potential problems of reproducibility or manipulative effect.

Among all parameters, the ethanol factor had only a significant *P*-value ($P = 0.0077$). This result showed the effective impact of ethanol concentration on the inhibition of growth of *Staphylococcus aureus*.

Moreover, the standardized residuals analysis showed that the points were offset. This point underlines that some bias could exist.

Analysis of *P. aeruginosa* at D0

Seven formulas out of sixteen had no inhibition data. The R-squared was low given the values of the *P*-value and of Lack of fit. No main effects could be proved.

Analysis of *C. albicans* at D0

Eleven formulas out of sixteen had no inhibition data. Nevertheless, the model was not significant. The 'Model *F*-value' of 1.94 implied that the model was not significant in relation to the noise. Only the ethanol factor had a significant *P*-value (= 0.0751) meaning that ethanol had a real effect on growth inhibition. The 'Lack of Fit *F*-value' of 0.15 implied that it was not significant in relation to the pure error.

In the residues graphic (Fig. 1), the distribution of the tests indicates a good homogeneity of residues.

Analysis of *A. brasiliensis* at D0

The 16 formulas were taken into account for this analysis. The R^2 obtained was significant ($R^2 = 0.76$). A response surface 2FI model was used here. A good distribution of tests could be observed on the 'normal plot of residuals' in Fig. 2.

Nevertheless, values indicated a bias on the predicted values meaning that the model did not uniformly provide minimum and maximum values. Figure 2 in the second graphic indicates a good estimation of errors.

Finally, an effect between the two factors, ethanol and pH was demonstrated. Consequently, there was a synergistic effect of these two factors against the proliferation of this mold.

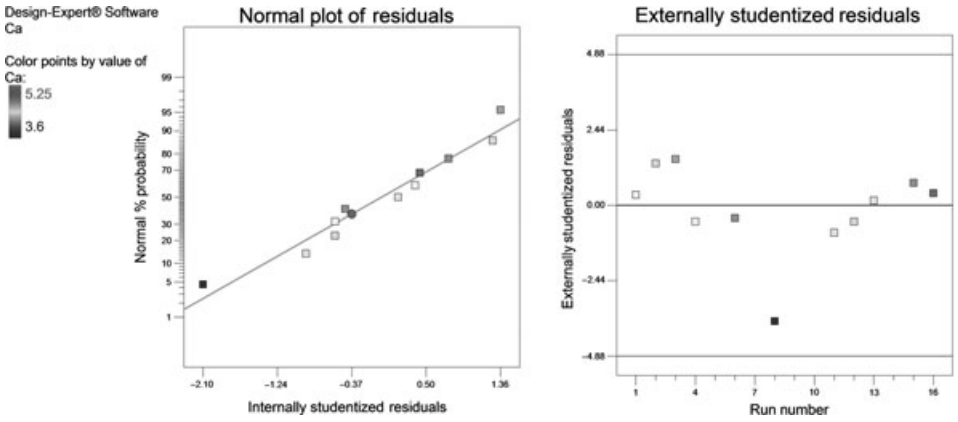

Figure 1 Right of Henry (left) and distribution of residues (right) for *Candida albicans* at D0, as drawn by Design Expert®.

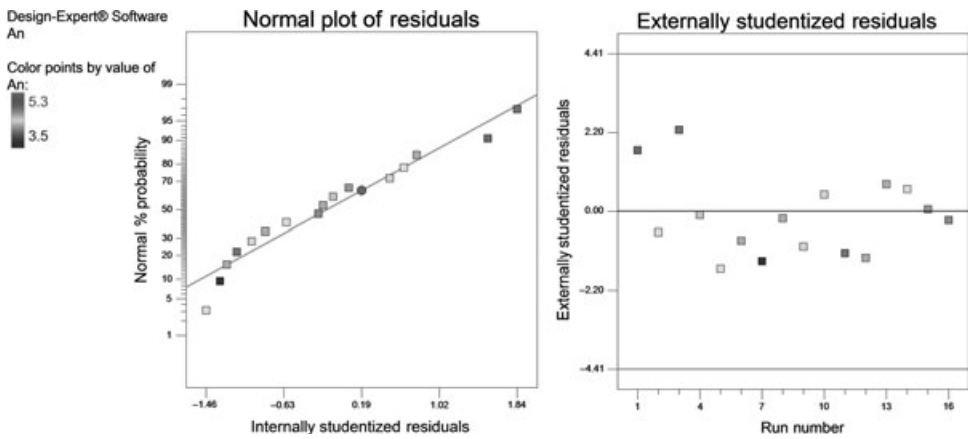

Figure 2 Right of Henry (left) and distribution of residues (right) for *Aspergillus brasiliensis* at D0, as drawn by Design Expert®.

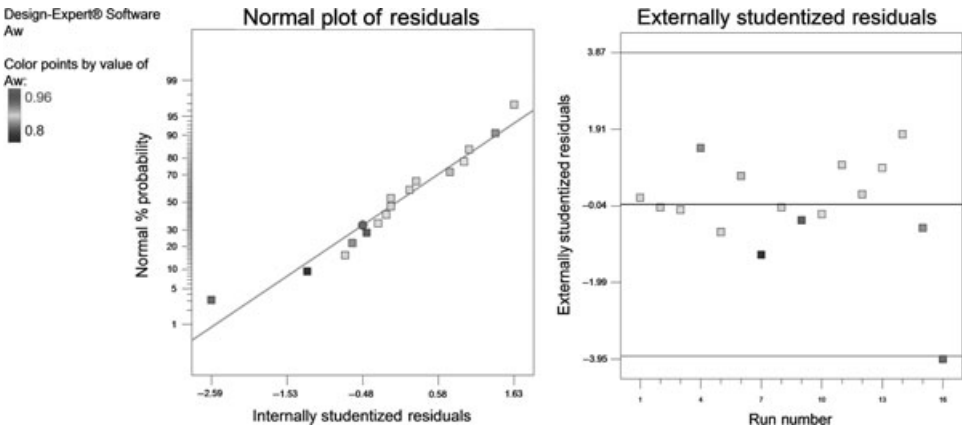

Figure 3 Right of Henry (left) and distribution of residues (right) for aw as drawn by Design Expert®.

General analysis at D7

All formulations adjusted at 11 decreased to a pH near to neutrality (7–8). Therefore, these formulations did not support this high level of pH. However, no variations for pH 4 or 7.5 were measured. Moreover, the a_w was evaluated but no variation was noticed.

Only formulas 4, 6, 11, 12 and 15 were contaminated. For formulations 4 and 15 (which was a replicate), this could be explained by the low level of ethanol, pH and glycerin. Concerning formulation 6 which had a pH of 11, this could be explained by the drop in the pH.

For formulas 11 and 12, growths were observed only for *Aspergillus niger*. This can comfort the hypothesis that this mold is more sensitive to ethanol than it is to pH and glycerin. Only the formulas at 4% of ethanol presented contamination by this mold.

Data are not available with Design Expert® because of lack of values.

General analysis at D14 and D28

Contaminations at D7 decreased at D14 until D28 but two remained still important. Indeed, formulas 6 and 11 were still contaminated by *Aspergillus brasiliensis*. The main reason for this was the drop of pH from 11 to 7 as well as the low level of ethanol. The concentration of glycerin had no influence in that case.

Data are not available with Design Expert® because of the lack of values.

Analysis of a_w

Therefore, a_w was not a distributed parameter, as it could not be possible to monitor. Therefore, this factor was evaluated at the end of the formulation and was thus considered as a specific response factor.

The concentration of ethanol, glycerin and the pH could influence this factor. Ergo, the relation between these parameters and the a_w was evaluated with Design Expert®.

The R-squared value was significant ($R^2 = 0{,}9927$) and the ANOVA analysis showed that the system was highly significant given the *P*-value of < 0.0001. The residual analysis (Fig. 3) showed a good uniformity of points with the ovoid shape of the set of points.

The analysis of the error distribution (lack of fit test) showed no significance in terms of residual errors when compared with outright errors. This proved that the model was properly adapted.

Table VII Presentation of the global data of the study

Parameters	Effects on development of strains
Ethanol	Important effects of inhibition with 20%.
pH	No significant effect for a single parameter from 4 to 7.5
a_w	Lack of growth despite an a_w greater than 0.6
Ethanol and pH	Synergistic effects on the antimicrobial conservation

In Fig. 3, the red dot is the negative residual of greater amplitude. It is usual that the point minimum and maximum of a series does not fit the straight line.

The analysis model for the a_w was very significant and therefore it was possible to establish a linear relationship between the four factors.

The relation between a_w and the three parameters can be modeled by the following mathematical equation:

$$a_w = 0.9737 - 0.0040 * \text{Ethanol} - 0.0010 * \text{pH} - 0.0036 * \text{Glycerin}$$

Final analysis

Finally, it was possible to establish the first elements of a predictive microbiology model as in the food industry [12, 14, 15, 18] but a further study is needed to refine the data.

Hereafter is the Table VII that summarizes the global data:

For combined parameters, only pH and ethanol showed a significant effect for *A. niger* at D0, especially with a high concentration of ethanol and extreme pH.

This article showed a different approach to the study of the challenge test in relation to previous works. Indeed, our experimental design was based on the variation of several factors without preservatives unlike previous studies, which studied different combinations of preservatives in the formula. [4]

In addition, this report discusses new parameters (as pH, a_w, ethanol) of variations from other studies conducted to date. [2]

Conclusion

Thanks to an experimental design approach using 16 formulations, the effectiveness of the three selected parameters was evaluated. The formulations used in our study could support wide variations of ethanol concentration, pH values and glycerin concentration without noticeable effects. Indeed, the results of the CT reveal that most of combinations offer good preservative conditions and the most of the formulas (14 out of 16) meet the International standard ISO 11930: 2012 criteria A. In the conditions of the study, the determination of a single parameter could not guarantee a microbiological conservation. However, a high concentration of ethanol associated with an extreme pH could inhibit bacterial growth from the first day (D0). A further observation was that a high concentration of glycerin influenced the product aspect and could influence microbial growth.

Currently, the ISO 29621:2010 standard 'Guidelines for the risk assessment and identification of microbiologically low-risk products' aims at helping cosmetic manufacturers and regulatory authorities to determine the finished products with a low risk of microbial contamination [19]. The standard describes the general characteristics including those hostile to microorganisms and discloses that the water activity (a_w) should be under 0.6.

From our study, it appears that despite an a_w above 0.6 (even 0.8) and without any preservatives incorporated in the formulas, it is possible to guarantee the microbiological stability of the cosmetic product when maintaining the right combination of the selected parameters.

Moreover, from the analysis of the different values obtained during the experimentation, it seems that there is a correlation between the a_w and the selected parameters cited above. An application of this relationship could be to approximate the a_w without any dedicated equipment.

Nevertheless, as the study focused on the range limits of the parameter values used in the experimental plan, some biases emerged in the challenge test results. A further study is needed to evaluate the conservation of products with parameter values selected in the range, e.g. to try formulas containing less ethanol and a pH less basic or acid, or to shift the experimental design. This would correspond more to the needs of industries and it would permit to observe a jump between compliance/non-compliance with regulatory criteria. Moreover, the future standard ISO 11930:2012 on 'Evaluation of the antimicrobial protection of a cosmetic product' could be used as the reference for the next study [20].

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