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MICROBIOLOGICAL INVESTIGATIONS AND REPLY OF DIFFERENT PRESERVATIVE SYSTEMS AND THE STABILITY OF PRESERVED EMULSIONS

FARKLI KORUYUCU SİSTEMLERİN MİKROBİYOLOJİK OLARAK İNCELENMESİ VE KORUYUCU İÇEREN EMİLSİYONLARIN STABİLİTESİ

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The stability of various emulsions containing different preservatives such as methyl paraben, nipa benzyl and lauric acid were studied Three emulsion types (w/o, o/w and w/o/w), prepared with exactly the same composition in order to avoid the influence of the formulation, have been studied. The degree of microbiological activities were tested with respect to Staphylococcus aureus ATCC 6538P, Pseudomonas aeruginosa, Klebsiella pnemonia CCM 2318, Esherichia coli ATCC 11230 and Candida albicans using the Agar Diffusion Cylinder Cup method. The systems were assessed by evaluating several parameters such as the macroscopic aspect, the droplet size and the accelerated stability under centrifugation and various temperatures (4, 25 and 40°C). Conductimetric analysis was used to confirm the types of the emulsions. It was found that the NML preservative mixture (containing methyl paraben, nipa benzyl and lauric acid) was the most effective combination on the test micro-organisms while the w/o/w emulsion system which was preserved with NM (containing nipa benzyl and methyl paraben) was the most stable formulation in stability studies. The reduced stability of emulsions containing NML mixture could be attributed to the insolubility of lauric acid in water.

Metil paraben, nipa benzil ve laurik asit gibi farklı koruyucular içeren emülsiyonların stabilitesi çalışıldı. Formülasyon etkisinden kaçınmak için aynı terkibi içeren üç farklı formül hazırlandı (sly, y/s ve s/y/s) Mikrobiyolojik aktivite, Staphylococcus aureus ATCC 6538P, Pseudomonas aeruginosa, Klebsiella pnemonia CCM 2318, Esherichia coli ATCC 11230 ve Candida albicans kullanarak, Agar Difüzyon Silindir Kap yöntemiyle test edildi. Makroskopik özellikler, partikül büyüklüğü, santrifüj ve hızlandırılmış stabilite testleri gibi parametreler değerlendirildi. Kondüktimetrik analiz ile emilsiyonun tipi kontrol edildi. Metil paraben, nipa benzil ve laurik asit karışımı (NML) en etkin koruyucu olarak saptandı. Metil paraben ve nipa benzil içeren (NM) s/y/s çoklu sistem en stabil formulasyon olarak bulundu. NML içeren emilsiyonların düşük stabilitelerinin, laurik asidin suda çözünmemesine bağlı olabileceği düşünüldü.

Keywords: *Simple emulsion; w/o/w multiple emulsion; Preservative efficacy; Stability*

Anahtar Kelimeler: *Basit emülsiyon; s/y/s çok fazlı emülsiyon; Koruyucu etkinliği; Stabilite*

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Introduction

Emulsions are the most popular delivery systems for applying cosmetics to the skin because of their several desirable characteristics. They permit the incorporation of many immisicible components in the same vehicle, have high consumer acceptance and allow widely different physical characteristics to be developed for cosmetics(1).

A cosmetic emulsion must be nonirritating and nonsensitizing, should be physically, chemically and microbiologically stable, must posses desired functional properties, should have desirable rheological properties, pleasant smell and appearance(2).

Microbiological considerations are the essential part of cosmetic formula development and evaluation. Preservatives as antimicrobial agents are widely used in cosmetics to protect the health of the consumer, as well as to maintain the potency and stability of the product(3). The objective of product preservation is to ensure the microbiologic safety and stability of cosmetics and drugs (4).

Stability tests are the final steps in the development of cosmetic products. Since cosmetics are mostly aqueous products, preservatives are added to formulations to prevent them from the growth of bacteria, yeasts and moulds. Consequently, preservative efficacy tests are performed on stored samples to evaluate the degree of protection with time(5).

The aim of this work was to examine the efficacy of different preservatives and their mixtures; to investigate their effects on different types of emulsions and to choose the most suitable preserved formulation. Moreover; the stability of these formulations were studied as reported previously(6). In this work the investigations were made on the w/o, o/w and w/o/w emulsions preserved with different mixtures of preservatives. The degree of antimicrobic activity was tested using Cylinder Cup Agar Diffusion method. The physical stabilities of each formulation were also studied.

Macroscopic aspects, the changes in particle size, centrifuge and the accelerated stability under elevated temperatures were employed as indicias of stability

Materials and composition

The following chemicals were used in formulation of the emulsions: The oil was paraffin oil (Birpa Laboratory, Turkey). The liphopilic surfactant was Abil EM 90®, a cetyl dimethicone copolyol (Goldschmid, France) and the hydrophilic surfactant was Synperonic PE/F 127®, an ethoxylated propylene oxide copolymer (ICI, France). The aloe concentrate was purchased from Johnson & Johnson, Turkey. The preservatives were lauric acid (Merck), methyl paraben (Nipa Hardwicker, Inc., USA) and nipa benzyl (Nipa, England). The hydrated magnesium sulphate ($MgSO_4 . 7H_2O$) was from Merck.

Culture medium: Mannitol Salt Agar, Biggy Agar, Saubaroud Maltose Agar, Nutrient Agar, Plate Count Agar, Malt Extract Agar, DRCM Broth, Asparajin Broth, Asetamid Broth, Selenit Sistin Broth, Brilliant Green Bile Broth were used as culture mediums as obtained from Merck.

Test micro-organisms: The test micro-organisms used were; *S.aureus* ATCC 6538P, *P.aeruginosa* (Faculty of Medicine, Ege University), *E.coli* ATCC 11230, *K.pneumonia* CCM 2318 and *C.albicans* (Faculty of Sciences, Ege University)

Preservative mixtures: The preservatives and their mixtures used were; methyl paraben (M) 0.5 $mg.ml^{-1}$; nipa benzyl (N) 0.5 $mg.ml^{-1}$; lauric acid (L) 5 $mg.ml^{-1}$; methyl paraben- lauric acid (ML) 0.5-5 $mg.ml^{-1}$; nipa benzyl- lauric acid (NL) 0.5-5 $mg.ml^{-1}$; nipa benzyl- methyl paraben (NM) 0.5-5 $mg.ml^{-1}$ and nipa benzyl- methyl paraben- lauric acid (NML) 0.5-0.5-5 $mg.ml^{-1}$, respectively.

Preparation of inoculum: Each microorganism was inoculated onto the surface of an appropriate nutrient agar medium from a revived culture of a freeze-dried stock sample. The bacterial cultures were incubated at 37 ± 1^0C for 24 hours and the culture of *C. albicans* at 27 ± 1^0C for 48 hours. Several subcultures were made to revive the microorganisms to their optimal state. The microorganisms grown were obtained by washing the culture medium with 0.1% (w/v) NaCl solution. Viable counts of the microorganisms in the microbial suspensions were carried out and the suspensions were further diluted with 0.1% (w/v) NaCl solution to give viable counts of approximately $1x10^8$ microorganisms per ml.

Microbiological test: About 20g of each accurately weighed, emulsion samples diluted 1 to 3 with 0.1% (w/v) NaCl solution (pH 7.2) were homogenized and centrifuged at 4000 rpm for 60 sec. The aqueous fraction was subjected to this procedure twice. The pooled solutions were stored at 4^0C (5,7). 1 ml of the pooled solution was mixed with 9 ml saline for dilution. One ml of all dilutions were surface plated on different medias and incubated at appropriate temperatures and days and the number of colonies were counted. The measurement of the plate count agar test was made after 24 h incubation at 37 ± 1^0C for the bacterial cultures and after 48 h at 27 ± 1^0C for the mould.

Preservative efficacy test was made by using the Agar Diffusion Cylinder Cup Method. In this method plates were poured with 20 ml base medium, containing approximately $1x10^8$ microorganisms per ml. We placed four Heatley cups (glass, l0x6 mm inner diameter and open at both ends) on each plate and filled these with solutions of preservative mixtures. The plates containing bacterial cultures were incubated at 37 ± 1^0C for 24 hours and that of the culture of *C.albicans* at 27 ± 1^0C for 48 hours after which the diameter of the zones were measured(8).

Method of Preparation of Emulsions: All the emulsions were prepared according to the same formula: 30% paraffin oil, 3.8% Abil EM 90®, 0.8% aloe concentrate, 0.7% magnesium sulfate, 1.2% Synperonic PE/F 127® and 63.5% distilled water, by weight.

The w/o/w emulsion was prepared in two steps and for the first step the w/o primary emulsion was formed at 2000 rpm for 20 minutes and then 80% of this was dispersed in aqueous solution of hydrophilic emulsifier at 800 rpm for 30 mins. The simple emulsions were prepared by adding the aqueous phase to the oil phase. In the case of w/o emulsion, both emulsifiers were introduced into the oil phase. In the case of o/w emulsion, each emulsifier was incorporated into the phase for which it had the greater affinity (9,10). The preservatives and their combinations were added into the primer aqueous phase.

Evaluation techniques

Macroscopic analysis: The main properties of an formulations were evaluated just after processing and were found to be very similar as; All the emulsions were white and homogenous creams with good consistency and their appearances were checked every day for a sign of phase separation and microbial contamination.

Microscopic analysis were carried out in order to follow the change of the droplet size of the emulsions over time. It was performed with an optical immersion microscope after diluting with the appropriate amount of the external phase (Carl Zeiss Jena).

Conductrimetric analysis were carried out to determine the emulsion type and the measurements were made at 20 ± 1^0C on the 1/20 diluted emulsion samples in an iso-osmotic glucose solution stirred magnetically (Jenway 4071 U.K.).

Stability tests: Stability was followed according to the analysis methods mentioned above. The freshly prepared emulsions containing preservatives such as methyl paraben, nipa benzyl and lauric acid were stored at different temperatures (4, 25 and 40^{0}C). The samples were controlled at specific time intervals for phase separation and particle size distribution. Centrifugation was performed at 3000 rpm for 15 minutes on fresh and 24 hour aged emulsions. The pHs of the systems were measured with a pH meter (Nel Mod 824).

Results and Discussion

Main characteristics of the emulsions: The characteristic parameters were given in table 1. Macroscopically all of the emulsions were white and homogenous creams. When diluted with liquid paraffin for w/o emulsion and with water for o/w and w/o/w emulsions homogenous mixtures have been obtained to show that the external phases were oil and water, respectively. The emulsion type was also confirmed by conductimetric analysis. Microscopic analysis have shown the presence of both the primary and the multiple characteristics (Table 1).

Table 1. Characteristics of the emulsions.

	o/w	w/o	w/o/w
Macroscopic analysis aspects	White cream	White cream	White cream
Consistency	Compact	Compact	Compact
Homogenity	+++	+++	+++
Microscopic analysis aqueous particles (μm)	1,5-2,5	1,5-3	6-9
Stabilitycentrifuge	Stable	Stable	Stable
pH	5,7	3	4,6
Conductivity	29,9	0.01	8.9
Continuous phase	Aqueous	Oily	Aqueous

Microbiological tests: Antimicrobial effectiveness was evaluated by the Agar-Diffusion Cylinder Cup method with different preservatives and their combinations. We studied the microbiological behaviour of the M, N, L preservatives individually and the mixtures NML, MN, ML and NL in order to establish the best synergic effect (Table 2). Comparing the preservatives individually, it was observed that methyl paraben (M) was effective on *C. albicans* and *S. aureus*, while nipa benzyl (N) was effective only on *C albicans*. However lauric acid (L) was found to be effective on all the microorganisms tested. The results of antimicrobial activity tests were found similar for ML and NL mixtures, but the NML combination was found as the most effective combination. The synergy of the NML association was verified by comparing its efficiency with the activity of each preservative used alone at the same concentrations (Table 2). Lauric acid is an unsaturated fatty oil and this marked difference which was seen in the L-combinations may be explained by synergy between components of the NML, ML and NL mixtures.

Table 2. The zone diameters of different preservatives and their mixtures for different micro-organism (μm).

Test micro-organisms	M	N	L	ML	NL	NM	NML
E.coli ATCC 11230	-	-	12	16	15	15	22
P.aeruginosa	-	-	15	16	16	12	20
K.pneumoniae CCM 2318	-	-	15	17	17	10	23
C.albicans	12	14	16	18	17	16	22
S.aureus ATCA 6538P	12	-	13	16	17	17	24

The microbial counts of bacteria, yeasts and moulds in unpreserved and preserved emulsions were shown in table 3. Primarily the microbial counts of unpreserved emulsions were realized.

Since NML was the most effective preservative combination on test microorganisms, it was used in the emulsion systems to determine the difference with unpreserved emulsion systems. The unpreserved emulsion systems contained high microbial counts (10^5-10^6 cfu g^{-1}) and this was not suitable according to the Cosmetic, Toiletry and Fragrance Association Inc. (CTFA) guideline. On the other hand the microbial count of preserved emulsions with NML (10-100) was found suitable according to the CTFA guideline which allows not more than 1000 cfu g^{-1} or ml^{-1} (11).

Table 3. Microbial counts of bacteria, yeasts and moulds isolated from unpreserved and preserved emulsion systems.

	o/w		w/o		w/o/w	
	Δ	0	Δ	0	Δ	0
TAPC	$1.4.10^6$	530	$1.5.10^5$	340	$4.4.10^5$	620
YMC	$1.9.10^6$	40	$5.9.10^5$	20	$1.4.10^6$	30

TAPC: Total aerobic plate count, YMC: Yeast and mould count, Δ = Unpreserved emulsion, 0 = Emulsion preserved with NML

The results obtained in the microbial investigation revealed that there was not a significant difference on microbial growth among the emulsions (Table 3). *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli* and *Candida albicans* were not found in the emulsion systems.

Stability studies: Stability of the systems is of great concern for all the emulsions. By centrifugation of the emulsions, the effect of the gravity would be accelerated. When the freshly prepared and 24h aged emulsions were centrifuged, no phase separation was detected except formulation NML in o/w emulsion.

An emulsion for cosmetic use has to be cutaneously tolerable but yet adequately efficacious (12). The most suitable pH interval is 3.0-5.0 in cosmetic systems. Therefore the pH s of the emulsions should be adjusted both for the appropriateness of the product to the users' skin and for its stability. Initial pHs of the emulsions were found as 3.0-5.7 in the present study (Table 1).

The thermal stability tests were done at 4, 25 and 40°C on three different emulsions containing different preservative systems (Table 4). The most stable formulations were the ones preserved with NM. w/o and w/o/w emulsions were more stable than o/w emulsion at 40°C and 4°C. In all the formulations, separation at 4°C was much higher than the others and this was in accordance with the results of Kallioinen et al(13).

The particle size measurements of the emulsions gave a good hint for their stability. The measurements of the size of the particles in w/o emulsions were shown in figure 1 and the average particle diameter of the fresh samples were 1.5-3 μm. Same tests were repeated after 15, 30, 45 and 60 days. Droplet size increased with time especially in NML emulsion proceeded by NL and ML with similarity. In NM emulsion, droplet size remained almost constant with time.

Table 4. Results of the thermal stability studies for the formulations containing different mixtures of preservatives

Preservative	Temp.(^{0}C) / Emulsion system	40^0	25^0	4^0
ML	o/w	3 weeks	>3 months	1 month
	w/o	1 month	>3 months	2 weeks
	w/o/w	1 month	>4 months	1 week
NL	o/w	2 months	>2 months	1 month
	w/o	45 days	>3 months	3 weeks
	w/o/w	1 month	>4 months	2 weeks
NM	o/w	4 months	>4 months	4 months
	w/o	4 months	>4 months	4 months
	w/o/w	4 months	>4 months	4 months
NML	o/w	1 week	>3 months	5 days
	w/o	1 month	>3 months	1 week
	w/o/w	45 days	>4 months	1 month

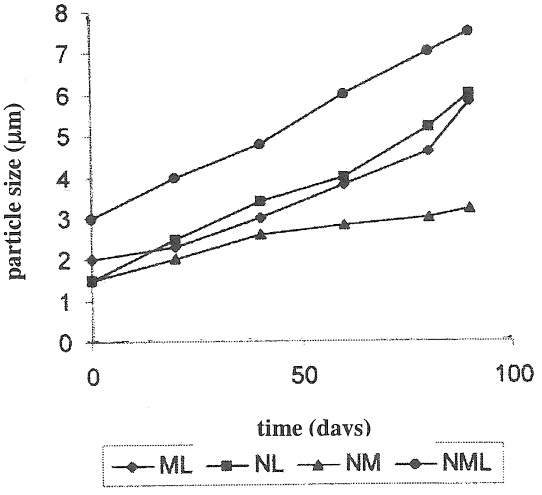

Fig. 1. Comparison of the particle size change versus time in w/o emulsions.

In figure 2 the size distribution of the particles in o/w emulsions is shown. The particles of the o/w simple emulsion showed a mean diameter of 1.5-2.5 µm. The increase in the size of the particles with time was found dramatically higher than that of w/o emulsions.

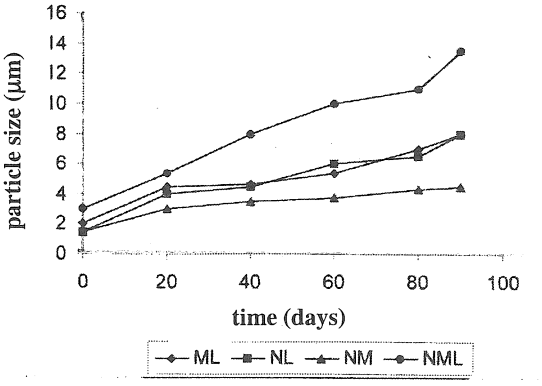

Fig. 2. Comparison of the particle size change versus time in o/w emulsions.

The measurements of the size of the multiple particles are given in figure 3. Their mean diameter was estimated to be 6-9 µm. The increase of particle size in w/o/w emulsions was almost constant with time.

At the end of 90 days it was observed that the most stable formulation was w/o/w emulsion system containing NM as it was found in thermal stability tests, as well. On the other hand all the

emulsions containing NML, which were the most effective mixtures on the test microorganisms were less stable comparing to the other formulations in the stability studies. The decrease of the NML stability with time could be explained by the insolubility of lauric acid in water(14).

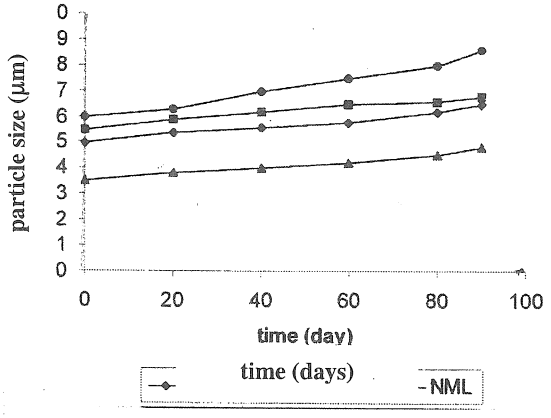

Fig. 3. Comparison of the particle size change versus time in w/o/w emulsions.

Conclusion

This study was carried on w/o, o/w and w/o/w emulsions containing different preservative mixtures. The main purpose was to prepare stable emulsions with different preservative mixtures. According to the preservative efficacy test it can be seen that the most effective mixture was NML. In the stability studies, it appeared clearly that the combination with lauric acid reduced the stability of the emulsions while it ensured the antimicrobial activity in preservative combinations because of its synergic effect. The emulsions preserved with NM were found to be the most stable formulations. Furthermore in all formulations, w/o/w, w/o and o/w emulsions were found stable, respectively.

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References

1. Semenzato,A., Bau, A.,Dall'aglio, C., Nicolini, M., Bettero, A.: Int. J. Cosmet. Sci. 16, 139 (1994)
2. Bhargava, H.N.: Drug Dev. Ind. Pharm. 13, 2363 (1987)
3. Kruijf, N.D., Schouten, A., Rijk, M.A.H., Pranoto-Soetardhi, L.A.: J.Chromotogr. 469, 317 (1989)
4. Orth, D.S., Kabara, J.J.: Cosmet. Toilet. 113, 51 (1998)
5. Semenzato, A., Benassi, C.A., Rossi, G., Bettero, A., Lucchiari, M., Cerini, R.: Int. J. Cosmet. Sci. 12, 265 (1990)
6. Özer, Ö., Baloğlu, E., Seiller, M., Ertan, G., Güneri, T.: Proceedings of the 20th Int. Fed. Soc. Cosm. Chem., Cannes, France, September 14-18 Abst no. 181, 1998
7. Benassi, C.A., Rettore, A., Semenzato, A., Bettero, A., Cerini, R.: Int. J. Cosmet. Sci. 10, 231 (1988)
8. Tuite, J.: Natural And Synthetic Culture Media For Fungi, (In) Rechigl, M. (Ed.) Handbook Series In Nutrition And Food, Section G: Diets Culture Media And Food Supplements, Vol III pp. 421-504 CRC Press INC. Clevelan, Ohio 1978
9. Ferreira, L.A.M., Seiller, M., Grossiord, J.L., Marty, J.P., Wepierre, J.: J. Control. Release 33, 349 (1995)
10. Ferreira, L.A.M., Doucet, J., Seiller, M., Grossiord, J.L., Marty J.P., Wepierre, J.: Int. J. Pharm. 121, 169 (1995)
11. Anelich, L.E., Korsten, L.: Int. J. Cosmet. Sci. 16, 25 (1996)
12. Tanmcı, N.: Deri Yaşlaması Ve Kozmetiklere Bir Bakış. (In) Hekimoğlu, S., Hmcal, A.(Eds) Kozmetoloji Günleri-1 pp.75-85 Şafak Matbaacılık Tic.Ltd.Şti. Ankara, Turkey 1996
13. Kallioinen, S., Helenius, K., Yliruusi, J.: Pharmazie 49, 500 (1994)
14. Kabara, J.: Medium- Chain Fatty Acids And Esters As Antimicrobial Agents (ln) Kabara, J. (Ed.) Cosmetic And Drug Preservation, Principles And Practice pp. 275-304, New York and Basel, Marcel Dekker, INC. 1984

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