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APPLICATION OF A FILM METHOD FOR MICROBIAL MONITORING OF COSMETIC RAW MATERIALS

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Synopsis

The possibility of using a film plate-count method, instead of the too complex and lengthy traditional methods, has been evaluated in cosmetic products. Petrifilm® is a ready-to-use system for detecting any microbial contamination. It consists of a flexible polypropylene film supporting on suitable dehydrated medium, a second support containing guar (a cold-water soluble gel-former) and an indicator on the internal surface.
Petrifilm® proved to have a good sensitivity and to be more convenient than the method recommended by the Italian National Pharmacopoeia. Results showed that it is possible to detect microbial and fungal contamination of raw materials of natural origin, which are frequently used in cosmetic preparations. Positive results were obtained on finished products (oil in water emulsions).

Riassunto

E' stata valutata la possibilità di utilizzare, per la determinazione della carica microbica di materie prime impiegate nei cosmetici e di prodotti finiti, un sistema pronto di conta in piastra (Petrifilm®, 3M). Esso comprende un adatto terreno di coltura su un film di polipropilene, un supporto contenente guar ed indicatore per evidenziare la crescita microbica. Il sistema ha mostrato una buona sensibilità e appare più conveniente rispetto al metodo tradizionale riportato nella Farmacopea Ufficiale Italiana. I risultati del lavoro, seppure preliminari, dimostrano la sua applicabilità nel controllo della contaminazione microbica di materie prime ampialmente usate nell'industria e delle emulsioni O/A.

Introduction

The criteria for microbial purity of cosmetic products are not exactly defined by Italian legislation. The national law 713 of 11.10.1986 states that cosmetic products "must not cause damage to human health when applied under normal conditions of use and should conform to criteria of microbial purity". These criteria have not yet been defined by the Minister for Health, so that recommended microbial limits and official control procedures are not yet available at present. The Minister's decree of 9.7.1987, reporting general instructions concerning manufacturing, handling and storage areas and equipments for manufacturing industries, states that containers and water utilized must be such as to avoid any risk of microbial contamination: a "qualified person" is also responsible for the microbial characteristics of the finished products.

The situation is expected to change rapidly, as the VI amendment (council directive 93/35/EEC) to the cosmetic council directive 76/768/EEC imposes the microbial analyses of raw materials and of the finished products for the protection of the consumer. While waiting for definitive instructions from the Public Administration it is possible to refer to the criteria recommended by the English and American associations, CTFA and CTPA, and to the proposed EC directive relating to microbial purity criteria which however has not yet been promulgated.

In this paper we have chosen as a reference the monograph in the Italian Pharmacopoeia (IX edition): "Microbial contamination of products not required to comply with the test for sterility". This monograph provides the relevant microbial standards and control procedures (1). The Pharmacopoeia test for the detection of microbial contamination consists in the traditional quantitative techniques for the count of bacteria, moulds and/or yeasts using agar media and qualitative tests for the absence of certain specified micro-organisms.

The execution of these tests is complex and lengthy, moreover it requires specialized personnel and suitable equipment both for the preparation of the test materials and for the execution itself. A more convenient procedure that would simplify and reduce analysis time is desiderable.

Petrifilm® is a ready-to-use system for detecting any microbial contamination. It consists of:

1. a suitable dehydrated medium (one of the two types: SM for total aerobic bacteria, YM for yeasts and moulds) supporting on a polypropylene film.
2. a second support containing guar (a cold-water soluble gel-former) and a growth indicator on the internal surface.

As growth indicator two reagents are used: tetrazolium for the total acrobic count in Petrifilm® SM or a phosphatase-sensitive indicator for the mould and the yeast count in Petrifilm® YM. The medium is rehydrated simply by inoculating the sample.

This system has already been shown to provide a valid alternative to the traditional methods in detecting microbial contamination in a wide variety of foods, e.g. fish (2), milk (3, 4), meat (5), cheese, poultry and frozen vegetables (6).

The Petrifilm® system has been accepted and adopted by AOAC to monitor milk and cheese products (7).

These results suggested the extension of these methods to other fields, such as the cosmetic industry.

The aim of the present work was to verify sensitivity and convenience of the Petrifilm® plate-count methods compared to a traditional plate-count method in routine analyses of raw materials used in cosmetic products. Preliminar analyses were conducted on finished products (oil in water emulsions).

Materials and methods

Materials

The materials tested are listed in **Table 1**.

Tests were performed on 17 vegetable or animal raw materials employed as functional substances in cosmetic products (4 of them being preservative-free) (nos. 1-17), on 8 plant glicolyc extracts (all by their nature containing preservative) (nos. 18-25), and on 7 commercial cosmetic products all of which were o/w emulsions (nos. 26-32).

Table 1

DESCRIPTION OF EXAMINED PRODUCTS

RAW MATERIALS

n°	**Functional substance**	**Preservative**
1	Soya seeds, specific fraction	Parabens; phenoxyethanol 0,8 %
2	Skin hydration factor	Not declared
3	Brain extract crusca	Propylenglycol
4	Hydrolyzed collagen	None
5	Vegetable polyoses	Parabens and phenoxyethanol 0,4-0,6 %
6	**S. cerevisiae** cell extract	Phenonip 0.5%
7	Calf thymus peptides	Phenonip 0.3%
8	Bovine ligament elastin	None
9	Bovine brain phospholipids	Phenonip 0.5%
10	**S. cerevisiae** oligopeptides	Parabens and phenoxyethanol 0,5%
11	Fish glycosaminoglycans	Phenonip 0.4%
12	Blood dialysate	None
13	Bovine brain liposomes	Phenonip 0.5%
14	Tyrosinase-inhibiting peptides	Phenonip 0.45-0.55%
15	Bovine heart extract containing Q. 10	None
16	Bovine brain liposomes	Not declared
17	Fibronectin derivatives	Not declared

GLYCOLIC EXTRACTS

18	Arundo donax	Propyleneglycol
19	Thymus vulgaris	Propyleneglycol
20	Urtica dioica	Propyleneglycol
21	Anthemis nobilis	Propyleneglycol
22	Rosmarinus officinalis	Propyleneglycol
23	Salvia officinalis	Propyleneglycol
24	Malva silvestris	Propyleneglycol
25	Opuntia ficus-indica	Propyleneglycol

EMULSIONS O/W

n°	**Main constituent**	**Preservative**
26	Not declared	Not declared
27	Jojoba oil-almond oil	Not declared
28	Glycerin	Not declared
29	Macadamia nut oil	Not declared
30	Tocopherol	Not declared
31	Vegetables extracts	Not declared
32	Vegetables extracts	Not declared

Table 2

MICROBIAL COUNTS (mean ± SE x 10^3 cfu/ml) AFTER SPECIFIC CONTAMINATION OF PRESERVATIVE-FREE RAW MATERIALS (Standard inocula of 10^4 to 10^6 /ml)

CONTAMINANT	PRODUCT (n°)		
	4	8	12
E.COLI			
TSA	1.4 ± 0,1	13 ± 0.1	2.5 ± 0.2*
PETRIFILM® SM	1.3 ± 0.1	14 ± 0.2	3.7 ± 0.3*
S.AUREUS			
TSA	4.8 ± 0.6	4.7 ± 0.8	4.6 ± 0.5
PETRIFILM® SM	6.4 ± 0.7	6.2 ± 0.2	4.2 ± 0.4
C.ALBICANS			
SDA+CAF	0.71 ± 0.5	1.6 ± 0.3	8.0 ± 0.1
PETRIFILM® YM	0.73 ± 2.4	2.7 ± 0.4	8.3 ± 2

*Statistically significant differences for p = 0.01

Microbial analyses for the detection of the total aerobic microbial and fungal counts

Initial samples were prepared by diluting 10 ml of each product with 90 ml of sterile peptone dilution water (Peptone Water, Difco). A suitable surfactant, 5% of sorbitan monooleate (Tween 80, Atlas Europol), was added to assist the suspension of poorly water-soluble products. If necessary, the pH of the dispersion was adjusted to about 7.

Samples 1,6,7,10 and 11 contained as preservative various parabens and phenoxyethanol (as shown in Table I). In this case microbial contamination was detected after inactivation of the preservative system by addition of 0.1% TRITON X-100 plus 0.5% TWEEN 80.

The analyses were carried out under conditions designed to avoid any accidental contamination of the samples, in a laminar airflow cabinet.

The conventional "pour plate" test for the total aerobic microbial and fungal count was performed by inoculating decimal serial dilutions of sample in Tryptone Soya Agar (TSA, Oxoid) and in Sabouraud Dextrose Agar (SDA, Oxoid) with added chloramphenicol (100 mg/l). Phosphate-buffered diluition water (Phosphate Buffered Saline, Dulbecco "A" Oxoid; pH 7.3) was used to prepare the samples. The plates for the total aerobic microbial count were incubated at 37 ± 1°C for 5 days, and those for the fungal count at 25 ± 1°C for 5 days.

According to instructions from the manufacturer, analyses by Petrifilm® method were performed by pipetting 1ml of the original sample and each of its serial decimal dilutions to a Petrifilm® Aerobic Count Plate (SM) and 1ml to a Petrifilm® Yeast and Mould Count Plate (YM). All plates were incubated at 32 ± 1°C or at 25 ± 1°C for 48 hours as appropriate. Analyses for all methods tested were performed in duplicate, three times each. Student's t test was applied.

Contamination Test

Because of the very low microbial contamination detected in all materials examined we decided to contaminate some of the raw materials prior to testing by inoculation of bacteria or yeasts.

Inocula of **Escherichia coli** (ATCC 25922), **Staphylococcus aureus** (ATCC 25923), **Pseudomonas aeruginosa** (ATCC 27853), a strain of **Candida albicans** clinically isolated, and **Bacillus spp.** isolated from no.18 were used.

Samples 4,8 and 12 of preservative free materials were contaminated with 10^4 to 10^6 micro-organisms/ml. Samples characterized by a self-preservative activity of solvent such as glycolic extracts were contaminated by an inoculum of about 10^3 to 10^4 spores per ml. Samples containing preservative systems (samples 6, 10, 11 and 26-32) were contaminated only after we had added TWEEN 80 and TRITON X-100 as neutralizers.

Results

Total aerobic microbial and fungal counts

All the samples examined except no. 18 showed total aerobic microbial and fungal counts of less than 10 cfu/ml. The total aerobic microbial count of no. 18 Provençal Cane, by both the "pour plate" technique in TSA and the Petrifilm® SM method, was 45 cfu/ml and 60cfu/ml respectively (no statistically significant difference). The contaminating bacterium was **Bacillus spp.**

Application of the contamination test to preservative-free raw materials

Microbial counts on antibacterial free samples 4, 8 and 12 after contamination by inoculation of standard bacterial suspensions (**Table 2**) were not significantly different in the two methods except for **E. coli** count in sample 12. Results show a higher sensitivity for Petrifilm©.

Application of the contamination test to glycolic extracts of plants

After contaminatiou of all glycolic extracts except no.18 with **Bacillus spp.**, we determined microbial counts by the two methods. Results were again not statistically different (**Table 3**) except for sample 19, where the Petrifilm® method was more sensitive.

Application of the contamination test to raw materials containing preservatives

Raw materials 6, 10 and 11, which contained a preservative system, were inoculated with standard suspensions of **E.coli** and **C.albicans** after neutralization of the preservative system. Again the results (**Table 4**) were identical.

Table 3

MICROBIAL COUNTS
(mean ± SE x 10^4 cfu/ml)
AFTER CONTAMINATION
OF NATURAL GLYCOLIC EXTRACT
WITH BACILLUS SPP.
(Standard inocula of 10^3 to 10^4 /ml)

Prod. (n°)	TSA	PETRIFILM® SM
19	3.3 ± 0.2*	5.2 ± 0.4*
20	0.29 ± 0.1	0.33 ± 0.7
21	3.8 ± 0.2	4.5 ± 0.2
22	0.32 ± 0.1	0.37 ± 0.9
23	0.45 ± 1	0.51 ± 1.1
24	3.8 ± 0.3	4.5 ± 0.3
25	3.2 ± 0.5	2.6 ± 0.4

*statistically significant differences for p=0.01

Application of the contamination test to o/w emulsions

After contamination of the selected emulsions, where the preservative system was neutralized, if necessary, (nos. 30-32), the microbial counts were not statistically different (**Table 5**) except for sample 28 iuoculated with **P. aeruginosa**. In this case the Petrifilm method was more sensitive.

Conclusions

The results show that the Petrifilm® system and the conventional methods showed no significant statistical differences except in two cases where the Petrifilm® method was the more sensitive.

The new procedure is more convenient than the traditional one in the following ways:

-Petrifilm® does not require preparation of the component parts requiring specialized staff or suitable equipment and requires less time for the procedure itself;

-it is a compact system requiring less space for sto-

Table 4

MICROBIAL COUNTS (mean ± SE x 10^5 cfu/ml) AFTER SPECIFIC CONTAMINATION OF PRESERVED RAW MATERIALS (Standard inocula of 10^4 to 10^6 /ml.)

CONTAMINANT	PRODUCT (n°)		
	6	10	11
E.COLI			
TSA	4.1 ± 0.8	3.6 ± 0.6	2.0 ± 0.3
PETRIFILM® SM	4.1 ± 0.7	3.6 ± 0.7	1.8 ± 0.2
C.ALBICANS			
SDA+CAF	42 ± 0.2	50 ± 0.8	50 ± 0.8
PETRIFILM® YM	43 ± 0.7	58 ± 0.8	52 ± 0.6

Table 5

MICROBIAL COUNTS (mean ± SE x 10^5 cfu/ml) AFTER CONTAMINATION OF EMULSIONS WITH STAPHYLOCOCCUS AUREUS AND PSEUDOMONAS AERUGINOSA (Standard inocula of 10^4 to 10^6 /ml)

CONTAMINANT	PRODUCT (n°)						
	26	27	28	29	30	31	32
S.AUREUS							
TSA	6.8 ± 1.3	14 ± 0.6	8.1 ± 1.0	15 ± 0.5	11	14 ± 0.8	7,4 ± 0.2
PETRIFILM® SM	7.5 ± 1.4	15 ± 2	7.9 ± 0.7	15 ± 1	11	15 ± 2	7,2 ± 0.4
P.AERUGINOSA							
TSA	26 ± 2	11 ± 3	19 ± 0.7*	20 ± 2	11 ± 2	8.3 ± 0. 7	15 ± 5
PETRIFILM® SM	27 ± 1	13 ± 2	24 ± 0.3*	27 ± 2	13 ± 1	11 ± 0.5	18 ± 6

*statistically significant differences for p=0.01

rage and during the incubation period.
-Petrifilm® plates are easier to read than agar plates. Sample n.18, for example, showed colonies wich became confluent in 24 hours in the traditional agar media, whereas in Petrifilm® plates colonies were always clearly defined and more easily identified. This last is expecially advantageous in the investigation of cosmetic powders wich often cause difficulties in colony detection because they alter the medium.
No incompatibilities among Petrifilm® and the raw materials tested were detected.
The Petrifilm® system is more economical if the total test cost is considered, because of the shorter time required to perform the procedure.
Further investigations have to be conducted especially on finished products; in fact, these preliminary data show the applicability of the method only to o/w emulsions.

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