COSMETICS MICROBIOLOGICAL ANALYSIS VALIDATION CERTIFICATE

We hereby certify that the following method and/or products:

The SEILAPARFUM protocol for the microbiological analysis of cosmetics (ref. MICROKIT PRT-SEILA-003, 31 pages) and the derived specifics for each parameter (PRT-COSM-001 to PRT-COSM-009, 30-38 pages each), the workflow diagram of which is attached for information purposes (not substituting for the aforementioned complete protocols), conducted using the latest available versions (MICROKIT references in brackets) of:

✓ **COSMETIKIT®** (KMT444), **COSMETIKIT®-EASY** (KMT448) and the described culture medium:

- ✓ **LPT Neutralizing Broth** (DMT217, RPL054, TPL053S),
- ✓ **LPT Neutralizing Agar** (DMT066, RPL074, TPL200),
- ✓ **Rosa Bengala Caf.Agar** (DMT101, RPL034, TPL072),
- ✓ **Cetrimide Agar** (DMT034, RPL010, TPL100, KMT476),
- ✓ **BCPT Agar** (DMT004, RPL024, TPL005, KMT477),
- ✓ **Mannitol Salt Agar** (DMT078, RPL023, TPL066, KMT480),
- ✓ **MugPlus Cfs.Agar** (DMT400, RPL444, TPL400, KMT479),
- ✓ **Biggy Nickerson Agar** (DMT017, RPL009, TPL062, KMT478) y
- ✓ **SPS Agar** (DMT116, BCD901, RPL039, RPL062, TPL089, TPL049),
- ✓ **CompactDryPlates® and Pathokit** (TC, YM, EC, XSA, KMT475),

meet the VALIDATION standards of UNE-EN-ISO 16140:2003, the results of which are attached. The validation has been performed by comparison, using quantitative certified and traceable strains versus official benchmark methods (Microbiology manual, the Cosmetology Advisory Committee of the Ministry of Health and Consumer Affairs, the Royal Spanish Pharmacopoeia and various technical standards in use or in ISO/UNE final approval stage for cosmetic microbiology).

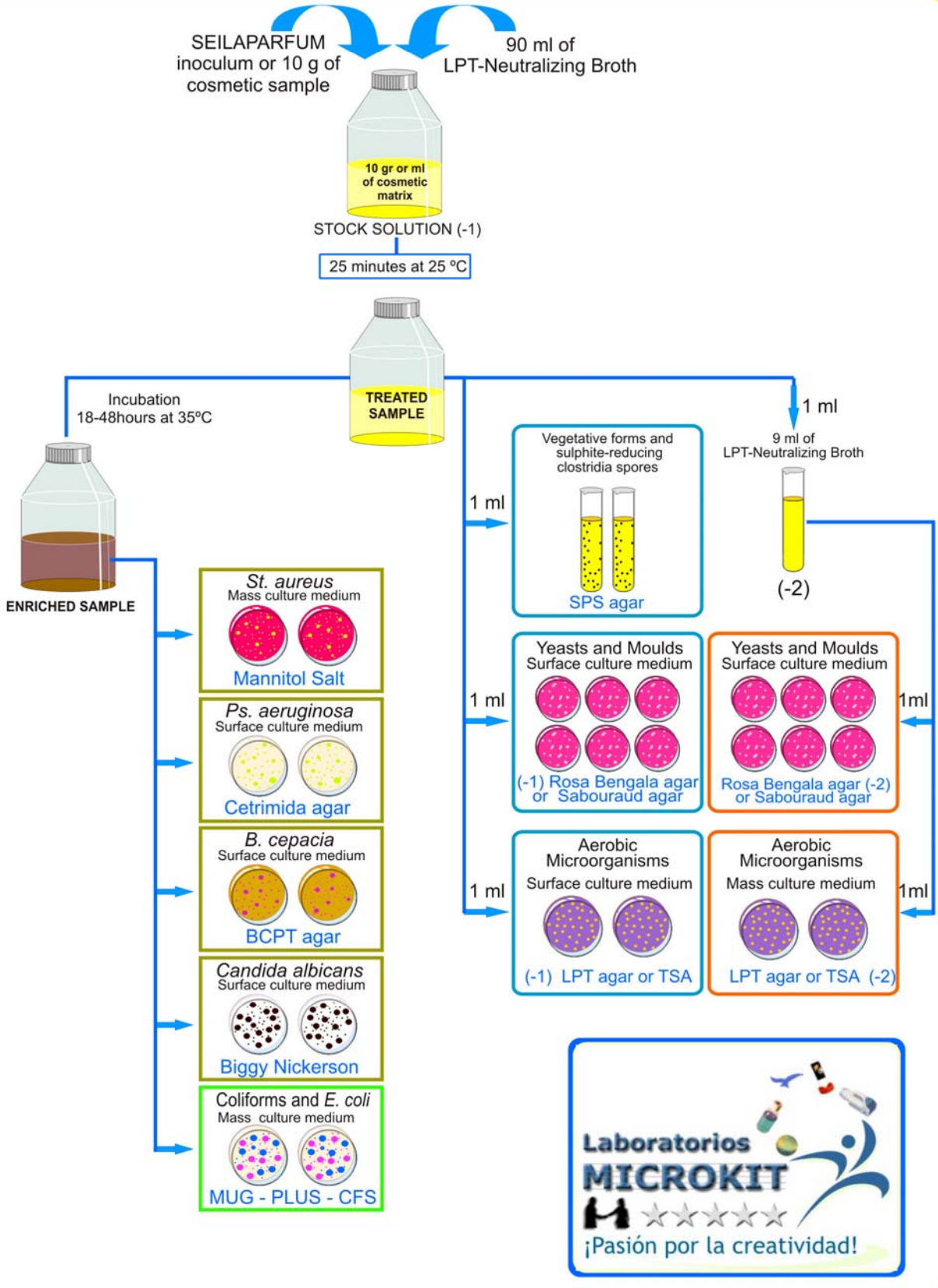
The present certificate is only valid for the period of validity of the cited methods and although it is guaranteed every four months by SEILA comparative revalidation, it must be renewed within five years from the date of issue indicated below. This certificate authorises the user of the method and the validated media to use the MICROKIT validation studies to endorse the internal validation or verification of their methods, media and kits with their own matrices, teams, analysts and in their own facilities, providing that they correctly use the methods and products referenced in and covered by this certificate, which cannot be extrapolated to other commercial brands.

Guaranteed by:

Date: 31March 2009
Last reviewed 25-09-2009

Jorge Sanchis Solera
SEILA Coordinator and MICROKIT® Quality Manager

DIAGRAM OF THE VALIDATED MICROKIT PROTOCOL FOR THE MICROBIOLOGICAL TESTING OF COSMETIC PRODUCTS



❖ VALIDATION METHOD

For each microbiological parameter, using a minimum of 16 and a maximum of 37 replications depending on the parameter, the presence/absence or the enumeration obtained using the MICROKIT method (PRT-SEILA-003 protocol and the derived protocols for each parameter PRT-COSM-001 PRT-COSM-002 PRT-COSM-003 PRT-COSM-004 PRT-COSM-005 PRT-COSM-006 PRT-COSM/AG-009) was compared to quantitative HPA certified strains. All the MICROKIT culture media described in the quoted protocols were used.

In addition, they were compared with results obtained in identical matrices with identical inocula by laboratories participating in the SEILAPARFUM comparative service that use the BENCHMARK METHOD (Pharmacopoeia and its Manual of Microbiological Testing of Cosmetic Products, 1994, published by the Ministry of Health and Consumer Affairs), which is used for the periodical revalidation of the MICROKIT method.

The MATRICES used were General Cosmetics, namely: massage lotions, anti- stretchmark creams, toothpaste, shower gel, shampoo, lip creams, tanning lotions, powder blushes, stick deodorants, infant wipes, mouth rinses and face tonics.
Restrictions of use of the MICROKIT protocol or media: None

❖ RESULTS

In blue, the MICROKIT results.
In black, the results of the benchmark method using the SEILAPARFUM comparison (third methods or participants that do not strictly apply the benchmark method are not included, for example those which count aerobics in plates using surface culture medium of 0.1, 3 x 0.33, 2 x 0.5 or 1 ml of stock solution and/or dilutions).
In green, the joint MICROKIT/benchmark method results:

1. RESULTS OF QUALITATIVE PARAMETERS

Inactivation and/or LPT Neutralizing Broth enrichment	Enrichment 48 hours at 35°C proved to be ideal for all subsequent detection:									
	SENSITIVITY (scarcity of False Negatives)				**SPECIFICITY** (scarcity of False Positives)				**DETECTION LIMIT FROM***:	
	MICROKIT METHOD		BENCHMARK METHOD		**MICROKIT** METHOD		BENCHMARK METHOD		**MICROKIT** METHOD	BENCHMARK METHOD
		%		%		%		%		
Coliforms/ *E.coli*	20 OK / 20	100	5 OK / 14	35.7	12 OK / 12	100	10 OK / 10	100	80 ufc/g *E.coli* 7ufc/g Colif.	80ufc/g *E.coli* 7ufc/g Colif
Ps.aeruginosa	19 OK / 19	100	11 OK / 22:	50%	15 OK / 15	100	7 OK / 7	100	7 ufc/g	40 ufc/g
Burkholderia cepacia	11 OK / 11	100	In Cetrimida 0 OK / 3:	0%	5 OK / 5	100	In Cetr. 1 OK / 1	100	70 ufc/g	For >250ufc/g
Staphylococcus aureus	22 OK / 22	100	In B.Parker 4 OK / 16	25%	12 OK / 12	100	In B.Parker 20 OK / 21	95	6 ufc/g	For >90 ufc/g
Candida albicans	14 OK / 14	100	9 OK / 11	82%	21 OK / 21	100	3 OK / 4	75	2 ufc/g	50 ufc/g
Salmonella spp.	3 OK / 3	100	--	--	34 OK / 34	100	---	--	2 ufc/g	---
Sulphite-reducing clostridia	7 OK / 7			100	24 OK / 24			100	90 ufc/g	
Enterobacteria	28 OK / 32			87.5	1 OK / 4			25	7 ufc/g	
Fungi (yeasts and moulds)	35 OK de 36	97	---	--	---	--	---	--	2 ufc/g	---

* N.B Microbiological uncertainty prevents us from asserting, in a statistically reliable manner, detection limits lower than those given. It will be noted that the MICROKIT method is much closer to the required detection limit than the benchmark method, except as regards coliforms and *E.coli*, which are the same for both methods. In the case of E.*coli, Burkholderia cepacia* and Sulphite-reducing clostridia, subsequent studies in matrices with inocula at lower levels will very probably show that the detection limit of the MICROKIT method is close to 1 ufc/g, as in the other parameters, all following the same 48-hour enrichment protocol of the initial suspension of the sample directly in LPT Neutralizing Broth, as such excellent results prove.

2. RESULTS TABLE OF QUALITATIVE PARAMETERS

Inactivation and/or Neutralizing Broth enrichment	LPT	Inactivation for 25 minutes at an ambient temperature 21-30°C proved to be ideal for subsequent counts:							
		ACCURACY				PRECISION (measured as Imprecision)			
		MICROKIT METHOD		BENCHMARK METHOD		**MICROKIT** METHOD		BENCHMARK METHOD	
Fungi (yeasts and moulds)		(Recovery 278 %). <+0.39 log and ±2 log accepted	OK	<±0.53 log and ±2 log accepted	OK	< ± 0.38 log and ±2 log accepted ¡0% out of range!	OK	< ± 0,59 log and ±2 log accepted 12% out of range	OK
Aerobics at 20-25°C		<0.58 log and ±2 log accepted	OK	<0.83 log and ±2 log accepted	OK	< ± 0.35 log and ±2 log accepted Only 4% out of range	OK	< ± 0.52 log and ±2 log accepted 9% out of range	OK
Aerobics at 30-35°C		<0.72 log and ±2 log accepted	OK	<0.82 log and ±2 log accepted	OK	< ± 0.44 log and ±2 log accepted 8% out of range	OK	< ± 0.56 log and ±2 log accepted 6% out of range	OK
Sulphite-reducing clostridia		(Recovery 167%) <+1log and ±2 log accepted			OK	< ± 1.1 log and ±2 log accepted			OK
Enterobacteria		(Recovery 69%) < -1log and ±2 log accepted			OK	< ± 0.63 log and ±2 log accepted			OK

❖ CONCLUSIONS

1-The **MICROKIT method**, when followed to the letter and with MICROKIT culture medium, is infallible. In all cases, it detects the relevant concentrations of all tested microorganisms. In addition, they do so at least as well as the benchmark reference, demonstrating far higher sensitivity and closer proximity to the minimum necessary detection limit, as well as better strength, ease of use and economy than the benchmark method, when implemented in the various laboratories that apply them.

2- The use of MICROKIT **LPT Neutralizing Broth** as a substitute for Letheen Broth, Beerens and other commercial inactivating broths (or those formulated by laboratories for their own use) enables better results to be obtained in comparative samples of all types of cosmetic matrices (including toothpastes), both for enumeration and the detection of pathogens and indicators.

3- The Enterobacteria enumeration parameter is not adequate, since, as with food matrices, all existing commercial detection methods (VRBG, EE Broth) have very poor sensitivity, up to the point that more confirmed coliforms are detected in other medium (such as VRBA, EMB, MUGPLUS) than Enterobacteria in the former, when the opposite should be

the case, as all coliforms are Enterobacteria, but not all Enterobacteria are coliforms. The detection of Enterobacteria, where the MICROKIT and benchmark protocols coincide, proves to be insufficient in both cases (87.5 % sensitivity, 25% specificity), and for this reason, the parameter should not be used as a recommended parameter in the cosmetics industry and should be replaced instead by the **detection and count of Coliforms (not only *E.coli*)**, since out of the three indicators of sewage contamination, it is this that proves to be the most robust and to have suitable quality analysis parameters.

4- The absence of a routine search for the ***Burkholderia cepacia*** parameter is highly controversial. Although no-one explicitly requests for a search for its absence, the website of the Agencia Española del Medicamento (Spanish Drugs Agency) clearly shows that all cosmetics withdrawn from the market over the last few years for bacteriological reasons have been exclusively due to this emerging pathogen. For this reason, MICROKIT has designed a selective medium named BCPT Agar which is giving excellent results, as shown by the PCR molecular identification of the suspect colonies obtained by users in their natural samples. Seilaparfum and Cosmetikit ®, both from MICROKIT, have enabled a number of Spanish cosmetics laboratories to already start actively looking for this emerging pathogen. We are confident that, following this validation, the percentage will rise to 100%.

5- Another highly controversial parameter is ***Staphylococcus aureus***, for which we should forget that there is a medium called Baird Parker, which only proves to be suitable for foodstuffs with high associated loads, but with cosmetics, we declare it INVALID due to its continuous false negatives and recommend use of the Mannitol Hipersalino Agar from MICROKIT, due to its validation by Seilaparfum.

6- ***Pseudomonas aeruginosa*** is another controversial parameter. In this case, it is not as a result of the Cetrimida culture medium, which has proved to be highly suitable for cosmetic microbiology, but rather due to its incubation at 42°C (as performed in many laboratories due to a distortion of clinical methods, in which the strain is abundant and highly active), instead of at 35 °C – the correct temperature – although it is preferable to obtain false positives than false negatives.

7- The ***Candida albicans*** parameter is only controversial when modern chromogenic media are used, designed to differentiate types of Candida in clinical samples, since it is very well detected by laboratories in the classic Biggy Nickerson.

8-It has been observed that some laboratories seed **surface or mass culture medium** indiscriminately according to whether the commercial culture medium that they acquire are prepared plates or prepared tube vials, when the rigorous standards of microbiology require that we seed mass culture when looking for fermentative microorganisms and aerobics, such as the majority of yeasts, *E.coli* coliforms and *Staphylococcus aureus*, and seed surface culture when we are looking for extreme aerophilics, as with some yeasts including *Candida albicans* and all moulds, thus including *Pseudomonas aeruginosa* and *Burkholderia cepacia*.

9-Attention needs to be drawn to the fact that some cosmetics laboratories **are still confined to the enumeration of aerobics and fungi**, unaware that virtually no pathogen (even after having been enriched in the appropriate broth) is capable of growing reliably in a culture medium intended for aerobic enumeration and that they are only part of the bacteria potentially present. Neither does *Candida albicans* necessarily grow well in fungi enumeration agars, since it has not been previously enriched for the count. If the pathogens are not actively searched for in the appropriate medium, they will be difficult to find, thus resulting in a dangerously false sense of security.

10-Another common, serious methodological error in laboratories that investigate pathogens, but do not participate in comparative services, is that they confine the initial treatment to 25 minutes of ambient temperature in neutralising broth (as is correct for subsequent counts of aerobics and fungi), but then **fail to enrich** the broth (or other) before isolating the pathogens on plates, resulting in the probability of finding the searched for pathogens being very low. For this reason, the **detection limit** of many laboratories is inappropriately high, as is shown in the Seilaparfum services in which these laboratories do not detect inoculated values of up to 70 ufc/g in some pathogens.

11-Few laboratories have proven routine use of **strains** to test that their method, reagents and analysts are working properly, thereby not detecting the critical points of their analysis in which test indicators may apply. Very few laboratories identify isolated colonies, which, we need to remember, are always presumptive in any solid culture medium, including highly selective, modern, chromogenic medium. This results in the loss of highly important information about positive scarcity that would normally be obtained. Even fewer cosmetics laboratories validate their analytical methods with their samples, analysts and reagents, when the Health authorities actively require it and there are courses and consultancy available specifically on this issue, such as those provided by MICROKIT.

12-The use of prepared plates for aerobic enumeration should be prohibited, since they absorb 0.1 ml (up to 0.3) of the initial 10^{-1} dilution. This means that when there is 100 ufc/gram in the sample, the plate only detects, at best 1 (3) colonies and just in the maximum uncertainty range, when it should detect a minimum of 15-30 colonies per plate in order for the count to be statistically “correct”, rather than just “estimated”. This corresponds to1500-3000 ufc/g, which is far above the permitted limits (<100 ufc/g ó <1000 ufc/g). For this reason, mass seeding is compulsory and this leads many laboratories that seed 0.1 ml in a surface culture medium to believe that their product is highly inhibitory, when in fact it is the method they are using that is inhibiting.

13-The best option for **fungi count**, which suffers from the same contingency as aerobics as regards legal ranges, but which cannot be seeded in a mass culture medium due to the extreme aerophilia of the moulds, is to seed in a surface culture medium, 3 plates per sample, distributing between the 3 plates 1 millilitre of treated sample (dilution – 1) and then adding together the count result obtained from the three plates in order to express the result in ufc/ml.

14-An increasing number of laboratories are aware of the need to **shake immediately** before each stage of the fungi count, as the spores float in a matter of seconds, making a minimally precise detection difficult when the inocula is taken from the middle or the bottom of the broth.

15- There are at least **two, very different, main populations** of aerobics, namely the saprophyte flora associated with humans that logically grows best at 35°C, and the saprophyte flora NOT associated with humans (but with raw materials) that grows best at 21-25°C. The use of an intermediate temperature in a vain attempt to detect all aerobics in a product as inhibitory as a cosmetic, seems to us, at the very least, not very sensible, even though it is considered by obsolete microbiological cosmetic recommendations. In the microbiology of food, water and the environment (air and surfaces), nobody has ever thought of doing likewise and they all search for both populations at the respective optimal temperatures (21-25°C and 30-37°C). This ought to be the case with more determination in cosmetics, since it is the most inhibitory matrix of all those we have mentioned, requiring indicators that are easy to detect. Aerobics at 35°C are considered indicators of human confinement and aerobics at 22°C are considered indicators of an alternative flora, whose count is often 10 times higher than the first.

For this reason, the warning limits of the latter are normally 10 times more tolerant (higher) than those of the former.

16-Some laboratories count without **triplicating the plates**, or, if they do, they only send us half of the count obtained. This disqualifies them from inclusion in the comparative service, as their results mean that they can not be included as minimally reliable statistical methods. In addition, however, they are running high risks on a daily basis, providing results with little contrast that depend largely on chance.

17-Some laboratories still **express their results** in an inappropriate manner, since "uncountable", ">1000 ufc/g" or "<100 ufc/ml" are not acceptable expressions for a precise microbiological result.

18-The analysis of 1 gram or millilitre of product is highly insufficient in many cases and of minimum rigour if a standard protocol is used, since in such a case and due to the stock dilution, the count of aerobics and fungi is carried out at 0.1 g/ml, as is the search for each pathogen. For this reason, wherever possible, **10 g/ml** needs to be taken and dispersed in 90 ml of LPT Neutralising Broth, taking 10 ml of this for each analysis (at least in the case of each of the 5 pathogens). In any event, a sample as small as 1g/ml in such an inhibitory product as a cosmetic, is well below the minimum quality standards that are required to obtain a reasonable and sensible uncertainty.

19-It has been observed that seeding in a mass culture medium for inclusion in **warm agar** is a much more critical point than previously thought, since a number of laboratories (including those with technicians with decades of experience in microbiological analysis) rely on touch and do not wait until the medium is sufficiently cold, thus very often preventing the growth of the microorganisms present. These false negatives prove, also thanks to Seilaparfum, that they are totally eliminated with more modern methods of seeding in mass culture medium, without heat, such as Compact-Dry-Plates®. The method used by the laboratory that obtained the maximum rating in the 10th Seilaparfum service is, in all parameters, LPT Neutralizing Broth + **Compact-Dry-Plates ®** appropriate for each of the parameters. These excellent results were repeated in other Seilaparfum and in a joint study of 27 participating laboratories (with over 1000 samples compared), as a result of which we declare the method VALIDATED not only as AOAC and Microval has done for food, but also, by MICROKIT, for cosmetic products. The method used by another laboratory which also obtained and excellent rating was LPT Broth Purple + **Pathokit**, a kit designed and manufactured by Labortorios MICROKIT to complement the parameters for which Compact-Dry-Plates® do not exist, namely *Candida albicans, Pseudomonas aeruginosa* and *Burkholderia cepacia*.

20-Some cosmetics laboratories do not give **water** the extreme importance that it deserves as a fundamental raw material in their products, as a few Seilaparfum participants also take part in comparative services specialised in water samples, such as Seilagua ®, believing obtaining a good result in the final product analysis permits them to lower their guard as regards the most important critical point of all. A total count analysis in some waters is totally insufficient, especially with the emergence of *Burkholderia cepacia* as an assiduous component of aquatic biofilms. There is no other industrial sector in which we consider water control to be more necessary and it is precisely in this area that there is no legal standard obliging laboratories to perform tests, relegating cosmetics to the forgotten industry as regards microbiological legislation.

21-Most Spanish laboratories are frightened by the idea of participating in an comparative exercise such as Seilaparfum and **do not even make an attempt,** even though they are assured maximum confidentiality and it is recommended as the best quality control tool for

their analytical processes. In other sectors (such as water or food), this participation is compulsory (and is customary) on the part of any laboratory wishing to be authorised by the relevant authorities in our country. Unfortunately, once again, cosmetics are behind the rest.

22-Some laboratories participate once in Seilaparfum and when they see that the methods and parameters are much more complete than those they are used to, meaning they do not obtain good analytical results, **they do not register again**, thus losing out on any possibility of improving and testing the usefulness of updating their centuries-old methods and parameters.

23-Although not a general trend, some laboratories take part in Seilaparfum to meet the minimum requirements or out of sheer curiosity, and do not **implement any improvements** following the repeated analytical faults that are detected by this tool and the advice given in the report. However, most laboratories that assiduously take part in the Seilaparfum comparative service also prove the usefulness of this service, **by increasing their internal rating** due to the improvements they implement as a result of the critical points detected and the recommendations obtained in the four-monthly reports.

24-Cosmetics products are traditionally placed in one of two groups: *Category 1: cosmetics for children under the age of three, around the eyes and in mucose membranes* and *Category 2: other products.* It is our opinion that Category 1 should be enlarged to include **cosmetics likely to be used by imunocompromised people** (in old people's homes, hospitals, childcare centres, etc.), especially when the average age of the Spanish population is increasing at an alarming rate.