TESTS FOR USE OF BEE PRODUCTS IN COSMETICS

Maria Pilar FERNANDEZ ARROYO
SPAIN

Introduction

Some of the bee products have been used in cosmetics and medicine since long ago.

Honey was used as a medicine and in beauty creams in ancient Egypt even before Tutankamen epoch. But most successful it was in the XVIth and XVIIth centuries when it was recommended almost for all affections of human body.

Beeswax was the best means the first pharmaceutists used for lending consistency to cerates, pastes and pomades. At present, it is widely used in beauty creams, and as hair remover.

Royal jelly was used first in treatment of old age. Now it is used by radiologists and other people who are exposed to dangerous radiations, for regeneration of cells.

Bee venom has been and continues to be considered the best biological product in rheumatic affections.

Propolis is used as antiseptic mouth wash and in disinfectant solutions for topic use.

Pollen has been recently added to the group of bee products used in diets but its success was so fast that it may be now considered as ranking second after honey, for its importance.

At the beginning, pollen was used as a supplement of vitamins, essential amino-acids and minerals, because it contains 21 amino-acids, peptides, enzimes, coenzimes, all the vitamins known except B_{12}, desoxyriboses (preceding nucleic acids which control memory and all cell processes), carbohydrates, lecithine, and other biological factors. Both in pollen and in bees' body, 27 elements were identified: Na, K, Ni, Ti, V, Cr, P, Zr, Be, B, Zn, Pb, Ag, As, Sn, Ga, Sr, Ba, U, Si, Al, Mg, Mn, Mo, Cu, Ca, and Fe.

At present, pollen is used in cosmetics, as extract, in creams, beauty milk, and for sun-bathing solutions.

Cosmetic dermatology, general account

Experimental evidence exists that cosmetics have a direct pharmacological effect, although unintentional, entailed by the allergies and eczemas which they cause.

The increasingly wide use of cosmetics caused more skin affections, 36% of face dermatites having an allergic origin. The allergic dermatites are manifest as acute and subacute eczemas, eczematites, herpes, oedema and often also pruritus.

Worth mentioning is the fact that the nail polish causes a great number of dermatites which appear at distance — on the chin, neck, and mostly on eye lids.

Lip stick causes eczemas in the peribuccal zone ; sensitisations may be either slow and gradual, or abrupt. They are due to the colouring matters used, to excipients : beeswax, alcohol and perfume, or to the antioxidants used for avoiding rancidity.

Because cosmetic preparations are extensively and daily used, the rate of intolerance is small ; it is determined after the number of users, the sensitisation, toxic and fotodynamic characteristics af every component, in cosmetics-free zones, as well as after the number of people having professional contact.

Sensitisation may be caused by all components of a cosmetic product and in its turn causes all stages of eczemas or dermatites in any part of the body, particularly in the face (especially the soft orbital zones and the pre-auricular zone).

Fotodynamic effects appear in the skin exposed to light, and cause lasting hyperpigmentation. The seriousness of the reaction depends on the concentration of the chemical compound which causes them and the individual sensitiveness. With

allergies, the reaction depends on an immunological process in which concentration is not the major factor.

The skin reactions of intolerance are either toxic or allergic. The cause of the toxic reactions lies especially in two groups of usual components of cosmetics : colouring matters and perfumes whose composition and percentage usually are not mentioned on the label ; also in the water used for their preparation, which should be submitted to a bacteriologic test and it almost never is.

Considering the above mentioned and according to other authors — GLUSHKOV N. M., TRUBETSKA P. G., CARLSON, VELESCU and CIOCA, we mention two more tests necessary in preparing any of cosmetics, in addition to those described by VELESCU and CIOCA (1973).

The first is the a test of sensitiveness in order to identify the Arthus phenomenon ; rabbits or guinea pigs may be used. In one of the flanks from which hair has been previously shaved, a standard solution of the cosmetic preparation is injected subcutaneously. The injection is made daily, for ten days running, in the zone of the first one, and the intensity and extension of the inflammation are constantly compared. The result of the test depends on the genetic sensitiveness of the animal and therefore genetically standardised animals must be used.

The second is a bacteriologic test both of basic elements and of the finished product, as the bee products are of two fundamental types — glucides and organic nitrate compounds.

The first are, for the heterotrophic micro-organisms, the main source of carbon in the synthesis of their own carbohydrates, lipids or other cell components, thus modifying the original formula of the cosmetic preparation.

When a micro-organism comes across a nitrate compound (pollen, royal jelly, polypectides), the first reaction is a hydrolisis which may turn the compound into pectides with low mollecular weight and at last in amino-acids, which in their turn are used as a source of nitrogen — diminishing or even annulling the effect intended when the formula was devised.

Following numerous experiments, we established the following requirements :

1. Taking of samples
2. Preparation of the samples
3. Microbiologic examination.

1. Taking of samples must be made so as to be significant for the quantity of cosmetics produced, at least 3,000 samples being recommended to be taken, evenly distributed during the packaging process (at the beginning, in the middle, and at the end).

The samples are kept in the laboratory at 2—10°C temperature.

2. Preparation of samples. It is very important to have a uniform sample.

The cosmetics may be divided into two large groups :

1. Liquids, emulsions or other easily suspendable products, for which we use diluents with 1% emulsifier (tween 80).

2. Creams, pastes and other heavily suspendable products, for whose dilution we use isopropyl myristate.

The mother solution must be prepared in a sterile recipient ; one part of cosmetic product and nine parts of diluent which is homogenized the best possible. After determining the pH, it is adjusted down to the neutral point.

3. Microbiologic examination. We shall only mention the tests, with not too many details.

Total number of germs

1. Equal quantities of the mother solution and the successive dilutions are seeded two times on agar broth with yeasts extract with pH = 7.2, melted and left to cool down to 45°C ; the seeded media are put into Petri dishes which are circularly stirred in order to homogenise them, and are left to cool.

2. When they are solidified, the dishes are introduced into an incubator at 35—37°C, and kept there for 48 hours, after which colonies are counted in every pair of dishes — the number must range between 30 and 300.

3. The total number of mesophylic aerobic bacteria for 1 g of cosmetic product is calculated on the basis of the average number found and the dilution used.

The test for *coli* organisms

1. The technique of dilution in selective liquid media according to the M.P.N system is used. As selective medium we recommend "CLVB" with pH = 7.2, in test glasses of cca 10 ml with Durham bell.

2. 1 ml of each dilution is seeded three times, and are kept in incubator at 34—37°C for 24 hours, or for 48 hours at the most.

3. Whether no gas is produced and no acid pH is recorded in the test glasses, it means that no *coli* organism exists in the sample.

4. Whether gas is produced and acid is recorded in one or more test glasses, the *coli* organism is determined according to the M.P.N. tables.

5. Of the test glass with the smallest quantity of seeded sample in which gas and acidity has been recorded, a "Lewine" medium is seeded with a platinum wire, and is kept in the incubator at 34—37% for 24 hours ; for the colonies with doubtful characteristics, INWIC is determined because together with Gram colour identification and morphological characteristics it confirms or not the existence of *E coli*.

Identification of pseudomonas

1. In a 250 ml flask containing 100 ml of non-selective liquid medium (soy and triptose broth with pH 7.2) 10 ml of the mother solution is seeded, after having been kept in incubator at 35°C for 24—48 hours, for the multiplication of the species of *Pseudomonas* genus :

2. Apparition of turbidity in the broth and more intensive pigmentation in the upper part confirm the existence of *Pseudomonas* species.

3. Of the previous test glasses two dishes are seeded with Agar King A (agar-pseudomonas-P) with a platinum wire, and two dishes with Agar King B (Agar-pseudomonas F) — each kept on different media — the first at 25° and the other two at 42°, for 24 hours.

4. Development at 42°C, production of piocyanine which is soluble in chloroform, and of fluorescein which is insoluble in chloroform but is soluble in water, as well as the positive reactions for cytochromoxidase und lecitinase, all show the existence of *Pseudomonas aeruginosa.*

5. In order to appreciate the concentration of *Pseudomonas* in the cosmetic product, when the result is positive, seedings with successive decimal dilutions are made, and according to the method described above the number of *Pseudomonas* for 1 g is calculated, in relation to the maximum dilution in which it was identified.

Determination of staphylococci

1. In a 250 ml flask containing 100 ml selective liquid medium (Chapman medium — mannitol) 10 ml of the mother solution is seeded, and is kept in incubator at 35—37°C for 24 hours, period which allows for multiplication of *Staphylococcus* genus.

2. Whether, after 24 hours a change in colour is noticed — from red to yellow, this means that *Staphylococcus* sp. exist.

3. For identifying *St. aureus*, the mother solution is carefully seeded with a platinum wire on dishes with Vagel-Jouhren medium with potassium tellurite, or on dishes with Baird-Paker medium with lithium chloride and potassium tellurite, after which they are kept in incubator at 35—37°C.

4. Whether by using these methods (V. Jouhren, or B. Parker) no suspect colonies develop in 48 hours it means that no pathogen germs of *Staphylococcus* exist.

5. Whether glittering black colonies with a whitish zone appear, this means that *Staphylococcus aureus* exists, and for confirmation coagulase, DNase, and phos-

phatase tests must be made ; they must be positive. The results of these tests must be considered together with the morphological characteristics and the colour affinity.

6. In order to determine the concentration of *Staphylococcus* in the cosmetic product, the initial process is completed by seeding successive dilutions on the culture medium, and continuing according to the indicated method, the total concentration for 1 g of cosmetic product is calculated, in relation to the maximum dilution in which the tests were positive.

Determination of streptococci

1. Rothe culture medium is prepared, selective for *Streptococcus*, and is poured into a 50 ml test tube with great diameter, and in four 9 ml test glasses.

2. In the large tube 10 ml of the mother solution is seeded, 1 ml is seeded in one of the thin tubes, and in each of the other three thin tubes — 1 ml of successively decreasing dilutions (1%, 1‰, and 1‰₀). All are kept in incubator at 35—37°C for 24 hours.

3. Whether colonies develop, without or with slight turbidity at the upper part of the medium and sediment on the bottom, this means that *Streptococci* exist.

4. In order to confirm their presence, a small quantity of the sediment is taken in a Pasteur pipette and spread on a slide, and Gram coloured, which enables us to identify the chain of Gram-positive streptococci. Also, catalase test is made, which must be negative.

5. The *Streptococcus* of *pyogenes*, *viridans* and *enterococcus* groups may be differentiated by the type of haemolysis on agar-blood dishes, and by the decarboxyline tyrosine reaction.

6. The concentration of *Streptococcus* is calculated in relation to the highest dilution in which the presence of the bacterium was recorded.

Determination of sporulated aerobic bacilli

1. 20 ml of mother solution is kept in water-bath at 70°C for 30 min in order to destroy the non-sporulated bacteria which might exist in the cosmetic product.

2. 50 ml of nutritive broth is introduced into a thick test tube, and 9 ml in four thinner tubes.

3. 1%, 1‰, and 1‰₀ dilutions of the cosmetic product are prepared from heated mother solution.

4. 10 ml of the standard dilution is seeded into the thick tube, 1 ml of it into the first thin tube, and 1 ml of the successive dilutions in the other tubes ; they are kept in incubator at 35—37°C for 48 hours.

5. Whether a colony develops, their motility, morphology, Gram affinity and number of spores must be recorded.

6. Dilutions are seeded on solid culture media (agar-broth and agar-blood), the tubes are kept in incubator, for the same period of time and at the same temperature. The morphologic characteristics, sporulation and haemolytic activity are recorded.

7. For final determination whether the sporulated bacillus is *Bacillus anthracis*, the pathogeny test on guinea pigs is made.

8. On the basis of the data recorded and of the material in the tube with the highest dilution, the presence of sporulated bacilli is confirmed with certainty. Then their concentration in one gram of cosmetic product is calculated.

Counting of fungi and yeasts

1. Three Petri dishes are necessary with OGA or Sabouraud culture medium, to which 40 units of penicillin and 40 micrograms of streptomycin for 1 ml, with pH = 5.5, are aded.

2. Dilutions of the mother solution are made — 1% and 1‰ — of cosmetic product.

3. With a sterile pipette, 0.1 ml of the mother solution is put on one of the dishes ; 0.1 ml of the 1% dilution of the cosmetic product on another dish, and 0.1 ml of the 1‰ dilution on the third dish. With a Drigalski pipette, the drop is

spread all over the surface of the medium, starting from the highest dilution to the lowest one, after which the dishes are kept in incubator at 22°C, for 5 days.

4. Whether colonies develop, their total number is recorded, and their number in one gram of cosmetic product is calculated.

Determination of pathogen fungi

1. Sabouraud medium is prepared in three dishes, to which chloramphenicol and actidion (1) are added — as they brake the development of bacteria and of most of fungi which do not cause dermic mycoses. The most important species of *Epidermophyton, Microsporum* and *Trichophyton* genera develop well on these media.

2. On three dishes with the chosen medium, 0.01 ml of the 1% and 1‰ dilutions respectively (prepared for the previous countings) is pipetted, and then is spread most uniformly possible by means of Drigalski pipette, starting with the dish on which the highest dilution had been spread.

3. The dishes are kept in incubator at 28—30°C for 5 days, in humid atmosphere.

4. The culture is examined macroscopically or by slightly magnifying it, under light, on both sides, and the morphologic characteristics of the colonies are recorded. Slides for microscopical examination are prepared as follows : a very clean slide is smoothly pressed against the suspect colony (without spreading it). The slide is taken by curved pincers and is placed with the impression against a drop of Amann colouring matter previously kept for a few seconds on a slightly heated slide, and then it is examined under a dry-lens microscope.

5. Whether the colonies are white — like cotton or like a dust where they come into contact with the air, and chromium yellow on the other side, and whether at microscopical examination microconidia with small spores (2—3 μ) and fusiform macroconidia with 8—10 regularly distributed spores are seen, then they belong to *Microsporum* genus.

6. Whether colonies are small, with granulous aspect, and light green on the other side, and under microscope pyriform or club-shaped macroconidia are seen, they belong to *Epidermophyton* genus.

7. Whether colonies are cotton white where they come into contact with air, and red or violaceous on the other side, and pencil-shaped macroconidia are seen under microscope, then they belong to *Trichophyton* genus.

Determination of pathogen yeasts

The tests concerning pathogen yeasts are mainly intended for identification of *Candida albicana* species.

1. Petri dishes with selective medium (M. Candida-oxoid) are prepared for development of species of *Candida* genus.

2. On three dishes with the respective medium, 0.1 ml of the mother solution — on one dish, and 0.1 ml of the 1% and 1‰ dilutions prepared for the previous test) are pipetted on each of the other two dishes. Then they are spread throughout the surface of the dishes.

3. The dishes are kept in incubator at 28—30°C, and their condition is recorded daily, starting after 48 hours.

4. The macroscopic characteristics of the colonies which developed are recorded, and slides are prepared just as those of fungi, then they are coloured according to Gram method and are examined under microscope.

5. Whether in the C-oxoid medium convex, creamy colonies, of grey-blakish colour and thin rays on them are seen, one can assume that *Candida* species exist.

6. Whether under the microscope, slightly oval yeasts, with or without buds are seen, either separate or grouped on several segments of pseudomycella, and are Gram positive, the above assumption is confirmed.

By cultivation on agar with maize flour, many and long pseudomycella are obtained.

7. For identifying the *albicans* species, the fermentation activity — with production of acid and gas is checked on glucose and maltose, never on lactose.

The test of preservative substances

Tests are made for tracing the preservative substances in cosmetics, by means of micro-organisms, namely of certain strains which had been checked with respect

to their sensitiveness to preservative substances, such as : *Staphylococcus aureus*, Oxford type, *Klebsiella pneumoniae*, and *Saccharomices cerevisiae*, in recent and active cultures (micro-organisms can be obtained from the official Control Centres in every country.

Bee products

Initially, bee products were used as food ; later on they were also used in medicine, purpose for which they are still used today. In this country, the Carulla-Vekar S. A. Laboratories produce a very efficient preparation with honey — Milrosina, for ulcerations of buccal cavity, which contains :

Natrium bicarbonate	1.25 g
Resorcine	1.25 g
Vitamin C (calcium ascorbate)	0.25 g
Glycerine, fluid rose extract, and honey — c.s.p.	25 g

Kessler S.L. Laboratories produce an antiseptic mouth wash containing propolis, zinc sulphonate and alcohol propolis extract. The wash called Vigordenta is haemostatic and bactericidal.

The S.A. Hispano Quimica Farmacéutica Laboratories use bee venom for production of Reumapront, a preparation recommended for rheumatism of the joints and of muscles, which contains :

Bee venom 90 units
Vanillilamide of nonylic acid 0.2%
Benzyl ester of nicotinic acid 1%
Barnil ester of salicilic acid 3%

We have conducted research in this branch of pharmacology and in cosmetics and perfected several formulae. In preparing them, we set out from water- and liposoluble pollen extracts, from which proteins has been removed. The products are least likely to cause allergies.

It is still impossible to obtain a product causing no allergy at all, because the allergies are influenced to a great extent by the individual sensitiveness, which sometimes is quite significant.

A good pomade for healing wounds and regenerating burns, for haemorrhoids, skin chaps, etc. is obtained from polyethylene glycol, pollen extract, and rectified honey. The pomade helps granulation of skin, and consequently its regeneration. That is why it is recommended for protecting babies from urine, thus avoiding diaper rash. It is also efficient in healing pruritus.

For nourishing the hand skin by fats, vitamins, and amino-acids, a creme is prepared, including beeswax, sorbitol, pollen extracts, methyl paraben and propyl paraben. It avoids red spots and chaps on hands, protects them from the effect of hot water and of detergents, providing them with fats and the nutritive elements which keep up skin elasticity. For best results, they must be used regularly.

Also with pollen, a nourishing creme for skin is prepared, contaning the substances necessary for perfect functioning of the cell metabolism. The creme contains beeswax, pollen extract, natrium borate and magnesium sulphate. Because all have a low molecular weight, they penetrate easily through epidermis, and by nourishing the derma they rejuvenate the epidermis.

For smoothing the skin excessively exposed to sun or to air, very efficient is a mixture of beeswax, propylene glycol, pollen extract, natrium borate and ammonium solution. It restores the elasticity and freshness of the dry and chapped skin, avoiding desquamation due to excessive irritation or dehydration.

Also, pollen extract is used, together with stearinic acid, sorbitol, and a number of esters of fat acids, for preparing a lotion for body, lotion which provides for the fats and oligoelements necessary for keeping the skin stretched and smooth, and for preventing the dead cells of epidermis from falling too early and from leaving the inner layers of the human body without protection.