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Wageningen, 14 oktober 1976.

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Analysis of cosmetics with regard to legislation.

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ter verkrijging van de graad van
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hoogleraar in de virologie,
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Throughout this thesis it was impossible to omit proprietary names and use exclusively scientific names of cosmetic ingredients. Mentioning these names does not imply any recommendation of the products to the exclusion of other similar products of other chemical supply houses.

Stellingen

Positieve lijsten van cosmetische grondstoffen geven slechts voldoende waarborg voor de veiligheid van cosmetica, indien tevens zuiverheidscriteria voor de genoemde stoffen zijn omschreven.

Dit proefschrift (I).

Etiketdeclaratie van cosmeticagrondstoffen moet zich beperken tot de "top-20 sensitizers".

U.S.Federal Register, 38, no.200, III.

Een negatieve lijst van een "monsterachtig" aantal verboden cosmetische grondstoffen moet drastisch worden gesaneerd, wil zij de aandacht van de overheid niet afleiden van de reëel mogelijke en voorkomende stoffen uit die lijst.

EEG-cosmetica-directive, bijlage II, (27 juli 1976).

Waarschuwingsteksten op verpakkingen van consumentenprodukten moeten integraal worden genormaliseerd.

Numerieke limieten voor microbiële contaminatie van waterhoudende cosmetica, zonder relatering aan zelfconserverende eigenschappen, geven geen voldoende bescherming tegen potentiële gevaren bij de consument.

De aanwezigheid van conserveermiddelen in talrijke compactpoeders en andere droge cosmetische produkten is niet noodzakelijk.

Het wettelijk vereiste gehalte aan eidooier in eishampoos kan alleen ter plaatse van de bereiding afdoende worden vastgesteld.

Cosmeticabesluit 1968.

Voor een goed overzicht van de benodigde methoden van onderzoek voor de Warenwet en voor de opstelling van analysetabellen, behorende bij de produkten van elk besluit, is het noodzakelijk om bij elk besluit de analytische probleemstelling te formuleren.

"Warenchemicus" (oktober 1973,blz. 162).

Voor een efficiente begeleiding van medische klachten ten gevolge van het gebruik van cosmetica is het noodzakelijk de distributeur wettelijk te verplichten de identiteit van de personen op te geven, die volledige informatie kunnen geven omtrent de samenstelling van de produkten.

Beroepenvoorlichting moet in een zo vroeg mogelijk stadium worden geintegreerd in de vakken van het leerpakket van het basis- en voortgezet onderwijs.

Proefschrift van D.H. Liem, 10 december 1976

Analysis of cosmetics with regard to legislation

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Summary.

A general picture of toxicological approach and practical aspects of cosmetic safety is described in this thesis. Such considerations are the basis for introducing negative and positive lists of cosmetic ingredients into cosmetic legislation. The first Dutch Cosmetic Act of 1968 already has several of these lists, but no analytical methods were given to control these lists. Therefore a study has been started with the aim to make analytical market surveys of several classes of potentially risk-bearing compounds. The analytical experience gained in the study can be used for the developments of official methods for the Dutch Cosmetic Act. The results of these market surveys can moreover be used as information on the actual use of risk-bearing compounds and will therefore contribute to the establishment of sound and significant cosmetic legislation in the Netherlands.

Eye make-up colours were identified by a set of characteristic reactions. Lipstick colours were identified by wellknown chromatographic methods. Colour intermediates for the oxidative hair colouring were identified by two-dimensionalthin-layer chromatography. The aromatic amines were confirmed by direct gaschromatographic analysis which permitted quantitative determination. A separation of phenolic intermediates by means of gaschromatography is also described. Suntan preparations were analysed for the presence of UV-absorbers, browning agents and local anesthesics. A simple aerosol sampling method, prior to gaschromatographic analysis was developed, thus permitting a total analysis of propellants and solvents in single-phase aerosols within an hour. Hormonal substances were detected in selected samples by chromatographic methods. These chemical findings supported the results of the biological assay for the detection of oestrogenic and androgenic activity of cosmetic products. Finally antimicrobial compounds were analysed in many kinds of cosmetic products, in which they were used for preservation or for its deodorizing, antidandruff or antiseptic actions. The diversity in chemical structure did not allow the development of universal methods, but most of the compounds could be identified and determined by chromatographic methods. Formaldehyde was determined by fluorometry. A study of the stability of formaldehyde releasing substances is presented.

Samenvatting

Een toxicologische benadering van de veiligheid van cosmetica is in dit proefschrift weergegeven. Een dergelijke beschouwing vormt de basis voor de invoering van negatieve en positieve lijsten in de cosmetische wetgeving. Het Cosmeticabesluit van 1968 bevat reeds verscheidene van deze lijsten. Voor de wettelijke controle van deze risico-dragende stoffen waren toen nog geen methoden van onderzoek beschikbaar. Daarom werd een begin gemaakt met een studie naar het voorkomen van deze risico-dragende stoffen in cosmetica door middel van marktsurveys. Niet alleen zou daaruit inzicht worden verkregen welke risico-dragende stoffen in werkelijkheid worden gebruikt -hetgeen een gezonde wetgeving zal bevorderen- maar tevens zou met de analytische ervaring een basis worden gelegd voor de ontwikkeling van officiële methoden van onderzoek.

Kleurstoffen van decoratieve oogcosmetica werden geidentificeerd door middel van een aantal specifieke sleutelreacties. De lippestift kleurstoffen werden langs dunnelaagchromatografische weg geidentificeerd. De componenten van oxydatieve haarkleurmiddelen werden geanalyseerd middels 2-dimensionale dunnelaagchromatografie en de identiteit bevestigd via de gaschromatografie. Tevens was het mogelijk de aromatische di-aminen en de fenolische verbindingen langs deze weg kwantitatief te bepalen. Anti-zonnebrand producten werden onderzocht op zonnefilters (UV absorberende stoffen), bruiningsmiddelen en locaal-anesthetica. Haarlak spuitbussen werden op de gebruikte oplos- en drijfmiddelen geanalyseerd. Daarbij werd een voorbemonsteringsmethode ontwikkeld ten behoeve van de gaschromatografische analyse. Hormonale stoffen in bepaalde cosmetica werden via chromatografische methoden opgespoord en kwantitatief bepaald. De chemisch-analytische resultaten voor oestrogene en androgene stoffen konden de biologische ijkingen van die producten bevestigen. Tot slot zijn vele soorten cosmetica onderzocht op de aanwezigheid van antimicrobiële stoffen (conserveermiddelen, bactericiden). Het was niet mogelijk om voor deze groep stoffen met uiteenlopende chemische structuren een universele methode op te stellen. De meeste stoffen konden echter op eenvoudige wijze via de dunnelaagchromatografie worden opgespoord en via gaschromatografie worden bepaald. Formaldehyde werd fluorimetrisch bepaald. Een studie van de stabiliteit van formaldehyde afsplitsende conserveermiddelen is in dit rapport weergegeven.

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1. Introduction

This study was made in order to provide the first Dutch Cosmetic Act of 1968 (Cosmeticabesluit,1968) with actual information on the use of different classes of risk-bearing compounds in marketed cosmetics by means of analytical market surveys, in order to contribute to the establishment of sound and meaningful cosmetic legislation in the Netherlands. Moreover, the experience gained in such a study will make a valuable contribution to the still rather unexplored field of cosmetic analysis.

Legislation of cosmetics has two principles. The first principle concerns an aspect of public health, namely the consumer must be protected from products which are, or which might be, injurious to health. This principle deals with the toxicological safety of cosmetics, which will be discussed under section 1.1 to 1.6. The second principle is the promotion of fair trade practices, and deals with the truthfulness of information regarding its efficacy, its composition and its contents. This will be discussed briefly under section 1.7.

1.1. Safety of cosmetics.

The safety of cosmetics has been a subject of serious discussions among consumers, manufacturers and the government for the last ten years (Proppe,197 Goulding,1972 ; Federal Register, 1972 ; Griepentrog 1973 ; Butler,1974 ; Reijnders, 1975). These discussions are still going on and it is hoped that these efforts will result in a harmonious legislation of cosmetics, in which the consumer will gain maximum health protection, the manufacturer will find minimum hindrance in his research and production, and finally that the government will be able to maintain optimal guidance on the safety of cosmetics.

Safety of cosmetics means the absence of hazards under the conditions of use. Such freedom from hazards cannot always be understood in absolute terms. With reference to the most frequently perceived experiences of locally sited adverse skin reactions, absolute safety is impossible. A cosmetic product might be considered as unsafe if such a contact dermatitis frequently occurs. The exact measure of such a frequency index is, however, difficult to define. Moreover the collection of adverse reaction data is difficult.

Safety of cosmetics might also be seen as freedom from systemic reactions for the consumer. In this respect the freedom from hazards must be interpreted in more

definite terms, even if such a product is used for a lifetime. By analogy with the "acceptable daily intake" concept for food additives (WHO, 1974), one can compare the blood level concentration of a suspected ingredient after percutaneous absorption and under actual conditions of use, with the "no-effect blood level" of the suspected compounds.

Safety of cosmetics might simply be connected with non-toxicity in the case where the product is accidentally swallowed. In toxicological terms an " acute oral toxicity " test (in terms of the LD_{50} on laboratory animals) will give a preliminary indication of the non-toxicity of a product (Poprzan et al.,1966).

A new kind of health hazard was recently recognized in the possible environmental danger of using fluorinated hydrocarbon propellants (FEA,1976). These compounds are relatively stable and can mix with the upper layers of the atmosphere. Unfortunately, they might then react with ozone and thus deplete the ozone levels appreciably, resulting in an increased incidence of cancer.

The scientific approach of safety described here has been considerably studied and extended during the last few years (Gloxhuber, 1967,1970; Rand,1972; Elias, 1974; Malten,1975 ; Giovachini, 1972,1975). Though one should be conscious of the very low incidence of serious deleterious effects in the long history of cosmetics, we cannot rely on this fact for their safeness, particularly as so many new synthetic compounds have been introduced into cosmetics, the toxicological dangers of which are not fully known. The hexachlorophene (Pines, 1972), thalidomide and more recently the vinylchloride (Federal Register, Oct.1974) reports are warnings to the cosmetic scientist.

Reasonable safeness of cosmetics should, however, be achieved somehow. The fact that a growing amount of toxicological research on cosmetic ingredients and on formulated products has been initiated - not least by the industry itself - during the last few years, is very promising and will result in filling in the gaps of basic toxicological data for the cosmetic ingredients and in setting up procedures for safety testing of the finished products. The results of these scientific efforts will also be a sound basis for any kind of regulations for cosmetics.

1.2. Toxicological approach to cosmetic safety.

Consideration was given in the previous section as to what "safe" might include. Most toxicologists take a two-step approach. The first step is the evaluation of "safe ingredients". A cosmetic product, which is composed of "safe ingredients", can be regarded as "probably safe". But it is possible that toxic responses of ingredients might alter - decrease or increase - by mutual interaction or by influencing several of the ingredients. A second step is therefore necessary, name-

ly the testing of the formulated product. There are also several other reasons why formulated products must still be tested. Of prime importance is the evaluation of percutaneous absorption of suspected and potentially risk-bearing compounds. Such studies can only be made on formulated products under simulated conditions of use. Finally microbiological contamination studies which might be related to certain health hazards, can only be made on the formulated products.

For ethical reasons most toxicological tests are performed on laboratory animals. All toxicologists, however, agree that several human tests are still necessary since the human body, the skin included, reacts differently from the or skin of the laboratory animals. In the interpretation of the lethal dose (expressed as LD_{50}) and also of the "no-effect blood level" of certain compounds, extrapolation of the results on animals to humans - say with a safety factor of 100 - is the only way to approach safety.

One great difficulty in the toxicological approach of safety, lies in the setting up of experimental procedures (protocols) for testing, which should be related to the actual conditions of use. In contrast to food, which is generally taken in by mouth, cosmetic products are used in many different ways. In the first place the part of the body to which the cosmetic is applied varies. Face creams are applied to the face; hair lotions to the hair and scalp; lipsticks, dentifrices and mouth washes come into contact with the mucous membranes. The duration also varies considerably. Hair shampoos and colourants are left on the hair for several minutes and are then rinsed off. But a lipstick remains on the lips for much longer and a night cream is left on the face all night. Finally the sensitivity of the skin must be considered. Mucous membranes (lips, oral cavity, eye area) and baby skin should be considered as being in more health danger than the healthy human skin. Table 1-I shows the greater possibilities of health danger in relation to these three parameters of cosmetic conditions of use.

By this system, 4 classes of cosmetics can be made with relation to actual possibilities of health danger during use.

There are, however, some general tests to be performed on all cosmetic ingredients. The "acute oral toxicity" test on several animal species is such a general test. It gives an idea of how toxic the ingredient is and what the apparent mode of action is. "Skin tolerance"studies have also been regarded as general studies for all cosmetic raw materials, for the obvious reason that the skin is the main operational area for cosmetics. They will include irritancy studies on intact and abraded skin with single and repeated applications as described by Draize & Alvarez (1949) and also sensitization studies according to Landsteiner & Jacobs (1935) or by the maximization test of Magnusson & Kligman (1970).

The more specific tests are related to the health hazard due to the specific condition of use. Materials which come or might come into contact with the mucous membrane, should be tested for "eye irritation" (usually on the rabbit's

TABLE 1-1. CLASSIFICATION OF COSMETIC PRODUCTS

with indications of greater possibilities of danger.

Hair	cleaning(shampoos)	o
	colouring	o
	bleaching	o
	waving	
	straightening	
	conditioning	
	care	o
	setting	o
	growth stimulant	o
	anti dandruff	
Face	moisturizing,care	o
	mask	o
	antiwrinkle	o
	bleaching	o
	cleansing	
	refreshing	
	shaving aids	o
	make-up	o
Eye	make-up	oo
	antiwrinkle	oo
	cleansing	o
	drops	oo
	refreshing	o
Lips	care	oo
	make-up,lipstick	oo
Mouth	refreshing	o
	teeth care	o
	anticaries	o
Body,hand	cleansing	o
	perfuming	oo
	moisturizing	oo
	anti-sunburn	oo
	anti-perspirant	o
	deodorizing	o
	colouring	oo
	indoor tanning	oo
	massage	oo
Nail	hardening	o
	extending	o
	colouring	o
	lacquer remover	
Foot	deodorizing	o
	antiperspirant	o
	refreshing	o
Baby	washing,cleaning	oo
	skin care	ooo
	disinfecting	oo
	moisturizing	ooo
Feminine	bust improvers	oo
	hygiene	oo

Each dot is an indication of a greater possibility of health danger, with regard to three parameters in the mode of use, namely: a. sensitivity of the skin (dots for baby skin and mucous mebranes); b. duration of stay (dots if longer than 30 minutes); c. area of applied surface (dots for areas larger than face).

By this sytem four classes of cosmetic products can be made in relation to these greater possibilities of danger:

I	Greatest possible danger	with 3 dots
II	Products	with 2 dots
III	Products	with 1 dot
IV	Products with least possible danger,	no dots

eye). They include ingredients for eye cosmetics as well as for shampoos, bubble baths, feminine hygiene products, lipsticks, dentifrices and mouthwashes. "Inhalation toxicity" should be studied for all ingredients of aerosol cosmetics and dusting powders (Troy,1974 ; Giovachini, 1975). "Phototoxicity" and "photosensitization" studies should be performed on materials that might be used outdoors and which might be strongly irradiated by sunlight with the possibility of the formation of toxic degradation products.

Finally, systemic "medium and long-term biological studies" might be necessary for certain classes of suspected ingredients (WHO-technical report 539, 1974), for instance antimicrobials, sunscreens, hair colour intermediates (see Burnett et al.,1975), hormonal compounds and synthetic organic colours. Studies should be made giving special attention to metabolism, organ and blood damage, the influence on the reproductive system (including embryotoxicity and teratogenicity) , to mutagenicity and carcinogenicity. Where possible "no-effect blood levels" should be determined.

The test procedures described above refer to basic toxicological information about ingredients, which will be considered here as the first of the two-step approach for cosmetic safety.

The next step is the safety evaluation of the formulated product. Human studies for skin tolerance can be made, as described by Finkelstein et al.(1963) or by using a modified procedure described by Uttley & van Abbé (1973). More important are the studies of "percutaneous absorption" of suspected compounds from the formulated product (Grasso & Lansdown, 1972; Jungermann & Silberman, 1972). Conditions of use have to be simulated and the percutaneous absorption studied using radioactive labelled compounds. The actual blood levels obtained during the experiment can be compared with the "no-effect blood level", obtained from basic toxicological studies. Finally, studies on microbiological contamination which might be related to certain health hazards, can also be made on the formulated product.

However, the described ways of evaluating and achieving the safety of cosmetics do not end here. The tests are predominantly predictive in character. Actual safety studies must be made with the marketed product. Relevant consumer observations of adverse reactions on a particular product should be studied by the medical profession and, in particular, by dermatologists. A minimal frequency of relevant adverse reactions will prove the predicted safety.

The scientific approach described in this section is summarized in Table 1-II.

TABLE 1-II. APPROACH OF COSMETIC SAFETY

INGREDIENTS

Basic toxicological information of ingredients from animal studies.

General studies necessary for all ingredients

Acute oral toxicity on several animal species, icl. LD_{50} rat, etc.

Skin tolerance studies: irritation, on rabbit, single and repeated application.

sensitization, on cavia, Landsteiner test, maximization test.

Special studies, depending on modes of use

Eye irritation: on rabbit's eye; for ingredients which come in contact with mucous membranes, e.g. ingredients for shampoos, bubble baths, feminine hygiene products(vaginal sprays), lipstick, mouthwash, dentifrice.

Inhalation toxicity: incl. LC_{50}; for ingredients of aerosols and dusting powders.

Phototoxicity and photosensitization: for ingredients of suntan products and for compounds structurally related to compounds with known photo-toxicity and sensitization.

Medium and long-term biological studies; for suspected compounds and with special attention paid to

Metabolism

Blood and organ damage

Reproduction, including embryotoxicity,teratogenicity,oestrogenicity

Mutagenicity and carcinogenicity

FORMULATED PRODUCTS

Safety studies of the formulated products from animal and human studies.

Human studies for skin tolerance.

Percutaneous absorption for products containing suspected compounds

Studies under actual conditions of use. Blood levels compared with no-effect levels from basic longterm studies.

Microbiological contamination in special relation to health hazards

MARKETED PRODUCTS

Studies on consumer perceptions of adverse reactions from marketed products. This is a study of the actual safety of the product.

1.3. Safety of ingredients.

The safety assessment of ingredients is the backbone for the formation of positive lists. The compilation of such a list of ingredients reasonably assessed as safe, however, is not easy to achieve. The following difficulties must be considered and a multistep approach scheduled (Table 1-III).

Too many new ingredients have been introduced in cosmetics without full consider their toxicological properties. Approximately 6000 ingredients (fragrance and flavor not included) have been compiled recently by the US cosmetic industry (CTFA, 1973). This number is still increasing as new synthetic and semi-synthetic compounds are constantly being introduced into cosmetics. It will reamin just an ideal, if all the gaps of toxicological information have to be filled in systematically. Such a tremendous task would not be justifiable in relation to its importance, because many ingredients are hardly used in manufacture and many others can easily be replaced by ingredients of which we have sufficient toxicological information. Therefore, a considerable reduction of the number of ingredients is necessary. If relevant manufacturing data of actual consumed raw materials in cosmetic production during the last few years could be made available, we would know which ingredients are in actual use, and which gaps in toxicological information have to be filled in. Such an approach to data compilation of actually consumed raw materials has been made recently by the US Food and Drug Administration in its "Voluntary Cosmetic Regulatory Program" (FDA, 1972), which is now in its fourth year. Computerized data from reported cosmetic ingredients statements of cosmetics produced in the US in 1974, has elaborated such a unique list of cosmetic ingredients, ranked by its frequency of use (Table 1-IV). Such a list should be extended to Europe and Japan in order to obtain a more general picture of the importance of cosmetic ingredients. An alternative approach of systematic reduction of the number of ingredients could be made by technological screening. It should be done per functional class of ingredients (see Table 1-V for an example of classification), first by tabulating the technological properties, followed by the assessment of the best ingredients in that particular group. For the red organic synthetic colours for instance, a decimal reduction can easily be made by tabulating data concerning different technological properties, such as: light and pH stability, brilliancy, compatibility etc. The more than 50 red colours mentioned in cosmetic literature (for instance : DFG, 1968) could then easily be reduced to approximately 5. Objections to such reductions can be expected from the manufacturers and ingredient suppliers, because of patent situations and trade secrets. But a reasonable solution should be forced in view of the importance of improving cosmetic safety.

Another difficulty is the complexity of composition of many ingredients, with

TABLE 1-III COSMETIC INGREDIENTS, REASONABLY ASSESSED AS SAFE.

A multistep approach for realization.

- o Compilation of cosmetic ingredients. Classification (example on Table 1-IV.)
- o Rational reduction (approximately decimal) of the number of ingredients by statistical and technological criteria.
- o Standard monographs with purity and composition requirements for the selected number of ingredients.
- o Test procedures (protocols) for safety testing per ingredient class
- o Compilation of available toxicity data of the selected number of ingredients.
- o Gap filling research work of additional toxicity data of the selected number of ingredients.
- o Assessment for acceptibility (or provisional acceptibility) for the selected number of ingredients, with special attention to effective and miximum limits of concentration, fields of application and useful label warnings for the consumer.
- o Compilation of classified positive lists (example on Table 1-X).

TABLE 1-IV CLASSIFICATION OF COSMETIC INGREDIENTS.

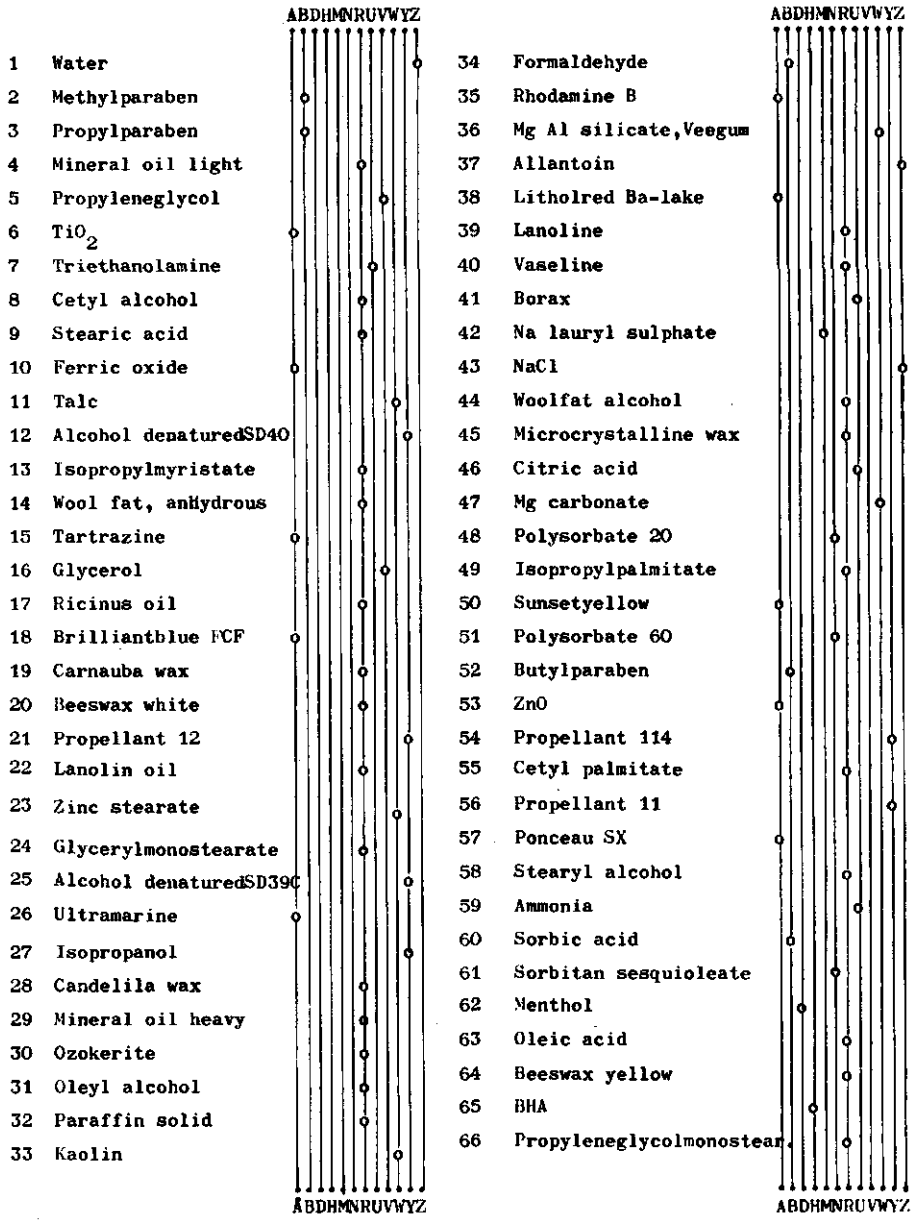
System used by the Government Food Control Station of Enschede.

A. Colours
B. Antimicrobials(preservatives included)
C. UV-absorbers(suncreens included)
D. Fragrance and flavor chemicals(essential oils included.
E. Oxidative hair colour intermediates.
F. Skin tanning and bleaching agents
G. Hair bleaching agents
H. Anti-oxidants.
I. Sweetening agents.
J. Hair waving and straightening agents
K. Agents for depilation.
L. Anti-perspirant agents
M. Anionic detergents
N. Nonionic detergents.
O. Cationic detergents.
P. Amphoteric detergents
Q. Insect repellants.
R. Oils, fats, waxes.
S. Anti-caries agents, fluorides.
T. Oxidation agents.
U. Acidic and alkaline agents. (alkanolamines included).
V. Skin moisturizing agents (humectants included).
W. Inorganic powders(talc included)
X. Polymers(thickeners included).
Y. Solvents and propellants.
Z. All other substances.

TABLE 1-V MOST FREQUENTLY USED COSMETIC INGREDIENTS (USA - 1974)

Reference: FDA,1975.

Capitals refer to classification of cosmetic ingredients of Table 1-1V



regard to impurities and the mixtures of several homologues and which differ from supplier to supplier. Impurities are the results of differences in raw materials or the results of different side reactions in the chemical transformation. These variations, which are very dependant of the chemical supply house, require standardization which can be defined for the most part in analytical standard monographs (for example : CTFA standards, 1971). Compilation of such standard monographs is an essential part of the realization of a list of cosmetic ingredients reasonably assessed as safe. This kind of work has been undertaken by several industrial groups at national level. International cooperation has been achieved by fragrance and flavour suppliers, namely the establishment of the RIFM (Research Institute of Fragrance Materials; see Opdyke,1976) in 1973. Because of the variability in composition of fragrance and flavor materials, their identity must first be defined before any toxicological experiments are started. The identity is defined by the standard specification of the EOA (Essential Oil Association) of the USA, with fingerprint-data from GLC, UV and IR curves, which will accompany any material used for toxicological research.

The next step is the setting up experimental procedures (protocols) for the safety testing of the different classes of ingredients in relation to their use. A guide for such procedures has been made by the Working Party on the possible toxicity of cosmetics of the Council of Europe (Council of Europe, 1975). A second example are the minimum test requirements of the RIFM (Research Institute of Fragrance Materials) as described by Opdyke (1976), and which consist of an acute oral LD_{50} in rats to see how toxic the material is; an acute dermal LD_{50} in rabbits to see whether toxic effects can be induced by penetration through the skin and how irritant the undiluted material is; a test for allergenicity to human skin by the repeated insult patch test or maximization test; a test of phototoxicity on the skin of bald mice, on swine and on humans, using natural sunlight and the solar UV simulator.

When all these safety testing procedures have been carried out, compilation of available toxicological information of the selected number of ingredients can be started. A lot of information can be expected from unpublished data of chemical suppliers. The gaps in information will become obvious and research into additional toxicological information can then be organized on an international basis.

With the availability of sufficient toxicological data, the assessment for acceptability can be made, with particular attention to the effective and maximum levels of concentration, the modes of application and the necessary label warnings. Compilation of positive lists of ingredients will then be possible. For practical reasons provisional positive lists can be compiled for materials of which only the most important toxicological information is available.

1.4. Safety of the formulated product.

In testing formulated products two possibilities exist: (a) the product is formulated from "safe" ingredients. (b) the product is formulated from ingredients of which we have insufficient toxicological information.

Products formulated from "safe" ingredients (a) need only additional testing (see Table 1-II). In particular a human test for skin tolerance should be made. More important are percutaneous absorption studies under simulated conditions of use. It is generally known that percutaneous absorption of pharmacologically active substances is very dependant on the vehicle of the product and on the condition of the skin.(Grasso & Lansdown, 1972). The blood levels found in such studies can be compared with "no-effect blood levels", obtained from basic long term biological studies. This aspect has been very neglected in the past, mainly because analytical techniques were tedious and unreliable. But with the development of versatile methods using radioactive-traced compounds, more studies of this kind have been undertaken during the last few years. One example is a study by Black & Howes (1975), which refers to the percutaneous absorption of triclosan from shampoos and deodorants. Penetration of ^{3}H-triclosan through rat skin under simulated conditions of use of the shampoo containing 0.05% triclosan was 0.197 µg per cm^2, and of the aerosol deodorant containing 0.1% triclosan 6.85 µg per cm^2. Concentration levels in the products and conditions of use seem to be important in determining the extent of penetration. As the permeability of rat skin is comparable to human scalp or axilla, these data can be used for human "no-effect blood levels" which were obtained from basic studies during a 3 week target organ study in rats, and which were of the order of 200 mg per kg per day. For a female consumer of 55 kg (area scalp = 1350 cm^2 and area axilla = 100 cm^2), the shampoo shows a penetration of 4.8 µg per kg, which is 42,000 times less than the "no-effect blood level". The deodorant when used twice daily will penetrate for 24.9 µg per kg, which is 8000 times the "no-effect level" in the target organ test. The safety of these products has been evaluated using this study of percutaneous absorption.

Products which are formulated from ingredients of which we have insufficient toxicological information, are more important for the present situation, because the compilation of "safe" ingredients is far from a reality. In these cases complete testing is required, which includes medium and long term biological studies. By testing the mixture of these ingredients, less time and less experiments are necessary to evaluate safety than if studies have to be made on the separate ingredients. On the other hand the conclusions from the safety experiments are only valid for the particular formula. An example of this type of testing has been published recently in the safety testing of oxidative hair colour-

ants (Burnett et al., 1975 ; Gloxhuber et al., 1972). Ito discovered in 1969 (see FDC-reports, 21 Sept.1970) carcinogenic properties of the meta-isomer of toluenediamine (2.4.toluenediamine), when rats were fed orally with the substance More recently Ames et al.(1975) and Harnden et al.(1975) have - almost at the same time - reported the mutagenic properties of these compounds on Salmonella species which might be related to carcinogenic properties. However, still further studies have to be made to see whether such a possibility really exists. If basic and long term biological studies of each of the ingredients of oxidative hair colourants (there are approximately 20 important compounds) would be made, such a task would take several years to obtain basic toxicological information. Because public opinion has pressed an answer, several studies of formulated products have been undertaken in order to get faster results. In one of such study Burnett et al. (1975) tested three hair dye formulations after mixing with hydrogen peroxide, thus simulating the actual conditions of use. The freshly mixed substance was tested for long term toxicity and carcinogenic activity by topical application to groups of 100 mice weekly or every alternate week for 18 months. None of the tested formulations produced evidence of systemic toxicity or carcinogenicity.

Finally, studies which can only be done on formulated products are microbiological contamination studies. They will be discussed separately in section 1.6.

1.5. Safety in actual use.

In the previous sections predictive safety has been discussed. Where the predictive testing ends, actual safety evaluation begins as soon as the products are used by the consumer. A full record of adverse reactions of each product marketed is generally regarded as necessary for judging the safety of a product. If such records prove that the frequency of adverse reactions of a particular product is relatively high compared with other products of the same class, such a product must be regarded as unsafe and therefore withdrawn.

These statistical measures of determining the frequency of adverse reactions for the different classes of products are, however, not yet available, so in a practical sense it is very difficult, or even impossible, to trace products which are dangerous to health. With the increasing use of new ingredients, the need for real safety standards becomes more urgent.

There are several possible pathways from which a complete record of adverse reactions can be compiled. Table 1-VI shows these possibilities. Such a compilation must be nationally centralized. In the Netherlands it would be practical to make the Government Food Control Station of Enschede, which has been appointed by the Minister as a specialized laboratory for cosmetics, into such a centre, for the obvious reason that analytical know-how and technological documentation of cosmetics are available there. Analytical guidance of incoming complaints are necessary,

not only to identify possible risk-bearing compounds in the product, but also as an essential aid to the medical profession in the diagnosis and therapy of the patient.

It is difficult to organize a national data bank for cosmetic complaints. In contrast to food poisoning, most cosmetic adverse reactions are mild and remain unnoticed except by the user. Some of the observed adverse reactions might be reported to public health authorities (in the Netherlands these might be any one of the 16 local Government Food Control Stations). Consumers can also go to the shop where the cosmetic was purchased and ask for a refund. In this case the complaint will probably reach the distributor or manufacturer. There is a possibility that records of products complaints are kept. If these distributors or manufacturers are willing to disclose these records to the proposed data bank of cosmetic complaints it would be an important contribution to the compilation of complaints. The third possibility is that due to the severity of the reaction, the consumer goes to the physician. If the skin reaction persists a dermatologist might be consulted. These medical records, as far as cosmetics are concerned, if channelled into the proposed data bank, would contribute to the compilation of the most important category of adverse reactions. All these possible pathways are summarized in Table 1-VI.

This compilation of adverse reactions has not yet been realized in any country, for the simple reason that all these possible pathways cannot be made mandatory. It all depends on the voluntary cooperation of everyone involved in the process, not least the consumer her or himself. In this connection it is interesting to mention a suggestion made by Aberg (1975) at a cosmetic symposium. He proposed attaching a small postcard to each cosmetic, on which the consumer - postage free- can note perceptible side effects and to send it to the public health authorities.

The most advanced approach towards establishing the frequency of adverse reactions has recently been made in the USA. In the Voluntary Cosmetic Regulatory Programme (FDA, 1972; see also Eiermann, 1976), manufacturers can cooperate with the Food and Drug Administration by a product experiences report. The lists(C) of the top-6 cosmetic products, most frequently cited in adverse reactions, is derived from such data (FDA, 1974), Table 1-VII. Two lists are presented. The ones on the left are ranked to the absolute amounts of recorded complaints and with no consideration of the numbers of items used by the consumers or produced by the manufacturers. The right list is ranked to the relative amount of recorded complaints in relation to the amount distributed or the number of users. This list gives an indication of which type of products might have the highest chance of giving adverse reactions.

It can easily be deduced from Table 1-VI which pathways have been used for the approach of these studies. The list (A) of Table 1-VII were made by path 2, and

TABLE 1-VI POSSIBLE PATHWAYS FOR CENTRALIZED COMPILATION OF COSMETIC COMPLAINTS

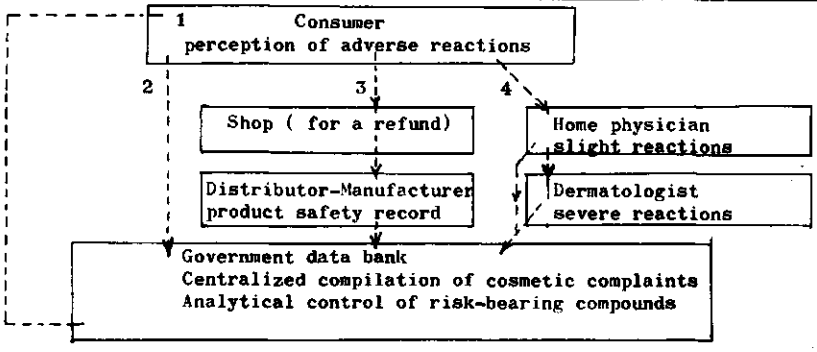


TABLE 1-VII TOP-6 COSMETIC PRODUCTS MOST FREQUENTLY CITED IN ADVERSE REACTIONS

(A) 1973 (USA) 525 complaints to Bureau of Compliance FDA Reference: Eiermann,1974	Ranked by % of total complaints received Deodorant-antiperspirant 12% Eye cosmetic 10% Shampoo 4% Intimate spray 2% Oxidative hair colour 2% Hair spray aerosol 2%	No data
(B) 1974 (USA) 3 months 589 complaints, confirmed by dermatologists from cross-section of 36,000 consumers Reference: Westat inc.,1975	Ranked by % of total complaints received Deodorant.antiperspirant 30% Soap 13% Cream-milk 12% Toilet water-perfume 8% Eye cosmetic 8% Hair spray aerosol 5%	Ranked by number of complaints per 10,000 users. Deodorant-antiperspirant 49 Depilatory 44 Cream-milk 17 Bubble bath 16 Hair spray aerosol 16 Oxidative hair colour 14
(C) 1974 (USA) 5305 complaints from manufacturer's records "Voluntary Cosmetic Regulation Program" Reference FDA, 1975	Ranked by % of total complaints received Cream-milk 21 % Oxidative hair colour 16 % Deodorant-antiperspirant 10 % Eye cosmetic 10 % Cold wave 5 % Shampoo 4 %	Ranked by number of experiences per 1,000,000 items. Depilatory 28 Cold wave 11 Cream-milk 9 Nail product 8 Oxidative hair colour 6 Deodorant-antiperspirant 4

are complaints made in 1974, from consumers all over the USA, and directly reported to the Office of Compliance of the Food and Drug Administration ; 525 complaints were recorded (Schaffner, 1975). The lists(B) of Table 1-VII were derived from the results of a three-months study (Westat,1975) of consumer adverse reactions, reported by a cross-section of consumers in the USA, involving 10,000 households and 36,000 participants. All complaints were verified by dermatologists. This "Westat" report chose path 1 of Table 1-VI. It is a unique contribution to the knowledge of the frequency of adverse reactions for cosmetics, because it involves many complaints, which usually remain unnoticed. The three-month project recorded 606 complaints. The lists(C) of Table 1-VI have been mentioned before. The experiences disclosed were reported to the US Voluntary Cosmetic Regulatory Programme in 1974, covering reports of 122 American manufacturers, including 80% of American produced cosmetics. This study follows pathway 3 of Table 1-VI. It is strange that path 4, which should be very important, has not yet been chosen in the USA, propably because of difficulties of cooperation with the medical profession.

The conclusions of these studies, in spite of the different sources of information are remarkably in agreement. This fact justifies the derived conclusions in the determination of the top-6 trouble causing cosmetic products. Deodorants and anti-perspirants head the top-6. This is an indication that antimicrobials are appreciable irritants. Further studies should be made to find ways of reducing this potential hazard. Depilatories and cold-wave preparations (both containing thioglycollic acid) are hardly considered as important if ranked to the total frequency of complaints (left hand data of Table 1-VII), but if ranked to the number of products (right hand data), these two products are top-irritants. A study of reducing the potential hazards of these two products is still recommended with particular consideration of the pH and the maximum admitted percentage of thioglycollic acid. Newly introduced ingredients for depilatories must also be suspected as a potential cause of adverse skin reactions. Skin creams and milks cause a surprising amount of trouble. A dermatological study with regard to the use of preservative and fragrance compounds in these products will be very useful. Surprisingly also are the hairsprays which cause many complaints. The potential causes should be identified and in particular the polymers, alkanolamines and the fragrance components.

1.6. Microbial contamination and health hazards.

A possible health hazard, which is not connected with the toxicological safety of ingredients, is microbial contamination. Before considering the health dangers, it is important to realize that human skin is very resistent to any kind

of bacterial attack. There might, however, be special conditions of the skin, in which bacterial attack can be dangerous. On a baby skin for instance during the first days of life, the application of certain amounts of Gram-negative bacilli will result in colonization. A proportion of the colonized infants will develop serious and often fatal generalized infections, including meningitis, which might be due to Flavobacterium meningosepticum (Parker,1971). Moreover, as long ago as 1901, Wasserman observed the spread of Pseudomonas aeruginosa in fatal infections in infants. Another suspected relationship between clinical infections and baby cosmetics was reported in New Zealand (Tremewan, 1946) and in Liverpool (Hills & Lederer, 1948). Deaths of several infants were possibly due to Clostridium tetani infections by contaminated talcum powder. These facts indicate that the baby skin is much more sensitive to bacterial attack than normal human skin of adults. Obviously higher microbial standards must be applied to baby cosmetics than to other classes of products.

Other conditions of the skin making it more vulnerable to infections are minor damages, such as is the case in acneous or eczematous conditions. As damaged skin might act as an entry point for microbes, the absence of pathogenic bacteria, yeasts and fungi should be a requirement in all kinds of cosmetics.

No reports are available in the long history of cosmetics stating that accidents have occured because of the direct use of microbiologically contaminated cosmetics. But the following reported cases will prove that there is a potential danger by contaminated products with Klebsiella species and Pseudomonas aeruginosa. Kallings et al.(1966) in Sweden described 8 cases of eye infections after the application of a corticosteroid-antibiotic eye ointment. It resulted in reduced visual acuity of the affected eye in 5 patients; in 2 cases the reduction was considerable and in 1 case the eye had to be enucleated. Some of the samples of the ointment contained more than 2000 Ps.aeruginosa per g.The potential danger of Ps.aeruginosa was studied by Marzulli et al.(1972), who were able to demonstrate that keratitis of the eye could be induced in laboratory animals (rabbit, monkey)by Ps.aeruginosa, but only to the damaged eye. Eye area cosmetics, shampoos and bubble baths, which can come into contact with the eye during use, should therefore be free from Pseud. aeruginosa. Such a standard is significant for the health protection of the consumer since the organism has been isolated many times from cosmetic products. Moreover, the consumer might often not be aware of minor damage to the corneal epithelium due for instance to the use of contact lenses because they are mostly invisible.

A second species of bacteria that has been reported as a cause of health problems is Klebsiella. In 1967 a hospital infection was reported in the Panama Canal zone, which was due to Klebsiella species and which had caused several cases of conjunctivitis. The hospital infection could be correlated to the use of hand lotions, which were contaminated with Klebsiella pneumoniae (Morse & Schonbeck, 1968).

The potential dangers described above, though not related to the direct use of cosmetics, can be considered as basic criteria for setting up microbial standards. Additional standards may also be considered in relation to hygienic conditions of manufacture and the hygienic state of raw materials. Such hygienic standards have been set up for many food products (dairy products, snacks etc). They are expressed in "total viable aerobic counts" per g of product , or "aerobic plate counts" per g, or also in the absence per g of an indicator organism. By monitoring the plate counts of products manufactured under well established hygienic conditions (or to use industry's term "GMP" = good manufacturing practices), such standards can be determined. However, at the same time, it is important to consider the preservative levels used in these systems, and to study possibilities of "over-preservation", which will create an unacceptable increase of another health hazard, namely the frequency of adverse skin reactions. The optimal preserving ability in a cosmetic product is a matter of "benefit against risk". Preservative levels cannot be held too low, because in general many re-infections will occur during use. Cosmetic systems should be made "self-sterilizing" and pass challenge tests after inoculating with artificial infection standards, including "in-house" organisms. The study of synergistic preservative systems is particularly useful , because such systems would allow lower concentration levels of preservatives, which would of course lower the incidence of adverse skin reactions.

There has been no general agreement of standard limits of total microbial counts for cosmetics. The World Health Organization in 1969 recommended a limit of 10^5 total viable counts per g, as a standard for non-sterile drugs (WHO, 1969), to which topical drugs (cosmetics included) can be considered. The US cosmetic industry has the following microbial limits as guidelines for cosmetics (CTFA, 1973): total viable aerobic counts not more than 500 per g for baby and eye area cosmetics and not more than 1000 per g for all other cosmetics. Discussions in the EEC, which are at an advanced state, have lead to the standards described in Table 1-VIII.

1.7. Fair trade practices.

Second to the"public health"principle of cosmetic legislation is the"fair trade practices"concept. This deals with the truthfulness, and non-misleading information , that the manufacturer passes on to the consumer with regard to the purchased product. The information includes not only labels and information pamphlets, but also information of the pre-purchased period, such as can be passed via the general mass media (radio, TV and newspapers), via counter-displays in the shops or as catalogues and information pamphlets via home mail. To simplify the problem, however, only package information (label, pamphlets) will be considered in this chapter.

Label information must bear a clear descriptive name of the product in order to

exclude any mistake in use. Sometimes a short description of the claimed cosmetic action can be stated. In these respects the manufacturer as well as the consumer has no controversial views with regard to the clarity of the information.

Manufacturers often claim a special cosmetic action. His product can do more than other competitive products or contains extra valuable ingredients. Obviously the consumer has to pay more for the product. The consumer, from his point of view, has the right to define his terms. In the first place he would like the special action to be explained in plain, clear and understandable language. Secondly, scientific documentation support has to be made available, in which that special action has been experimentally proved In the case of a claim to contain valuable ingredients, not only the name of the particular ingredient should be stated, but also the quantitative percentage, thus making public analytical control possible. These consumer requirements, though reasonable, are not easily fulfilled. In particular, the scientific proof of the special cosmetic action seems to be a hard burden on the manufacturer. A small glance at the abundance of cosmetic information pamphlets will make the problem clear. Special actions of the product are often described in poetical, lyrical and pseudo-scientific language, in many instances to camouflage the non-availability of scientific proof. This kind of information might suit pre-war generation of consumers, but at this time of mature consumerism such language is dissonant. What is required is honest, plain and truthful information. The question will be raised here, whether this problem in case of controversial views of the manufacturer and the consumer can be solved by rule making, for instance legislation. Cosmetic efficacy is in many instances very difficult to prove with sound scientific criteria. Endless discussions might be necessary for insignificant topics. A practical aid to reduce the proof for "special cosmetic action" is a system of"positive listing" of scientifically justified terms of cosmetic efficacy. Such a listing has been incorporated in Swiss and Japanese cosmetic legislation. An additional regulation can be made that in case the special action is not described in the list, the manufacturer should have experimental scientific proof at his disposal.

With relation to a manufacturers quantitative label statements of valuable ingredients, such an obligation can be made mandatory. This solution will not only satisfy the consumer, but it will also protect the manufacturer from unfair practices by his competitors.

Another problem is the question posed by the consumer as to whether a product is not overpriced. To make value comparisons, price as well as contents of the same product types of different brands vary considerably, which bears the consequence that the consumer is always forced to make a small (but not easy) calculation before making value comparisons. It is therefore more convenient for the consumer to have stated the price per g or ml on the label.

TABLE 1-VIII EEC-DRAFT PROPOSAL OF NUMERICAL MICROBIOLOGICAL LIMITS FOR COSMETICS Spring 1976. Reference (EEC,1976)

Sampling of 5 items of same batch code of marketed products.

There are the following possibilities to qualify the results. Judgment.

One +,or more than one +*	→	Fail*
Five ±*	→	Fail
Four ± and one -*	→	Fail
Three ± and two -	→	Fail
Two ± and three - (Eye or baby cosmetic)	→	Fail
Two ± and three - } Doubt* → Present*	→	Fail
One ± and four - } Doubt* → Absent*	→	Pass*
All -	→	Pass

* (+ means total viable count of 10^5 per ml or g, ore more; ± means total viable count between 10^3 - 10^5 per ml or g ; - means toal viable count of 10^3 per g or ml or less ;"fail" means "whole batch to be rejected; "pass" means whole batch accepted;"doubt" means that further tests should be made for specific organisms and also confirmed (Pseudomonas aeruginosa-Staphylococcus aureus - Candida albicans); "present" means present in 10^{-3}g ; "absent"means absent in 10^{-2}g)

TABLE 1-IX PRINCIPLES OF LISTING

Negative list: Compounds on the negative list are forbidden in cosmetics.Other compounds outside the list can be used in cosmetics.
Examples: EEC cosmetic directive Annexe II ; Dutch negative list; Swiss negative list.

Limiting list in negative sense: Compounds on this list are forbidden in cosmetics. except when used under specified limited conditions with regard to maximum levels of concentration, the field(s) of application and the labelling obligations. Other compounds outside the list can be used. Examples: EEC directive Annexe III-1; Dutch limiting list.

Positive list: Compounds on the list can be used in cosmetics. Other compounds outside the list are forbidden. For the sake of surveyance small positive lists of classified compounds are preferable to one big list, e.g. for colours, antimicrobials etc. Examples: EEC directive Annexe III-2; Dutch list of eye make-up colours; US colours for cosmetics; German list of colours (Mitt.III; reference DFG,1968).

Limiting list in positive sense: Compounds on this list and used below defined levels of concentration, can be used in cosmetics. Other compounds outside the list are forbidden. Examples: Dutch list of quaternary ammonium compounds; Swiss list of pharmacologically active compounds.

Mandatory labelling of ingredients has been introduced lately in the USA to facilitate value comparisons for the consumer (Federal Register,1974). In view of the complexity of cosmetic ingredient nomenclature, it is doubtful whether such value comparisons can be made in actual practice.

1.8. Principles of cosmetic legislation.

In projecting legislation for cosmetics, the principles of the three parties involved (consumer, manufacturer, government) should be built in as far as possible. In cases of controversial views the principle of the health protection for the consumer must prevail. Consumers ask for safety in use and truthfulness of product information. Manufacturers ask for minimum hindrance in research and production and for the safeguarding of their trade secrets. The government asks for sufficient executive powers to make the projected legislation significant and to be able to give optimal guidance in cases of adverse reactions or accidents.

What can be included in such a legislation will be systematically considered in the following:

(A). A sharp definition is necessary to indicate clearly which of the following boundery products should belong to cosmetics and which to pharmaceutics.

Cosmetics protecting the body: sunscreens, insect repellants.
Cosmetics preventing tooth decay: fluorinated toothpaste.
Cosmetics curing minor diseases:anti-acne, anti-dandruff cosmetics.
Cosmetics lightening the skin: skin-bleaching cosmetics.
Cosmetics decreasing perspiration: anti-perspirants.
Cosmetics deodorizing off-odours: deodorant, intimate (vaginal) sprays.
Cosmetics disinfecting the skin: medicated soaps, medicated sprays.

(B). The "harmlessness to health if used as directed" which is stated in almost any cosmetic legislation, is actually a toxicological statement. Though adopted by so many countries, it is peculiar that no country has further defined the toxicological criteria for the safety of a cosmetic product. Only Switzerland has made a small attempt by a correlation of the safety of a cosmetic product or ingredient to acute oral toxicity data in terms of the LD_{50} on laboratory animals. (Schweiz.Eidg.Gesundheitsamt, 1967). But in general, such a lack of further toxicological criteria for safety, has given the government a weak statutory position with regard to the proof of the harmfullness of the product. Only if a severe adverse reaction has occurred, has the harmfulness of such a product been proved. Such a statutory formulation with the aim of protecting the health of the consumer, has no prognostic value at all. Because of unexpected potential dangers, discovered in recent years, which have been correlated to the use of synthetic compounds (e.g. thalidomide, hexachlorophene, vinylchloride) and also

the fact that newly developed synthetic and semi-synthetic compounds in today's cosmetics is considerable, the discussion has been initiated as to whether it is necessary to transfer the burden of the proof for safety from the government to the manufacturer, which will then mean an equally statutory position for cosmetics as well as for pharmaceutics and pesticides. However, such a move will have far reaching consequences, such as mandatory registration with availability of toxicological reports including premarket testing. Such studies were started last year in the USA, but only for cosmetic products defined as drugs by the law, for instance sunscreens, deodorant-antiperspirants, medicated soaps, fluorinated toothpaste (Federal Register, 1972). There is also an alternative way of promoting the prognostic value of a projected legislation, namely the introduction of positive lists of"reasonably assessed as safe" ingredients (see under E of this section).

(C). Negative listing of injurious ingredients will in some way also prevent the marketing of harmful cosmetics, although it would be impossible to list all harmful compounds. Not only because of the existence of a large and ever increasing number of compounds, but also because of the lack of toxicological data for most of them, the idea of negative listing is no more than a desperate attempt to achieve cosmetic safety. The system of negative listing can be more usefully incorporated in legislation if done in a sensible way, namely to include only those compounds which have been of cosmetic interest in the past, but that have been proved to be harmful, and finally those components of complex ingredients, such as fragrance and flavour, that are regarded as toxic (including irritation and sensitization potential). These kind of complex ingredients cannot be satisfactorily regulated by positive listing.

(D). Partial negative listing of technologically indispensable but dangerous ingredients is necessary in cosmetic legislation. In such a case a limiting list in a negative sense (for principles of listing see Table 1-IX) can be compiled. The compounds of such a list can only be used under the specified restrictions (which are usually related to maximum concentration levels, the field(s) of application and the mandatory labelling of warnings). Examples of such limiting lists can be found in the Dutch Cosmetic Act (articles 2 and 3) and in the EEC cosmetic directive (Annexe III-1 and IV-1). These partial or limiting lists do not have a meaning in positive sense. If for instance formaldehyde is mentioned as a permitted preservative up to levels of 0.1%, the list does not prohibit or limit the use of other preservatives.

(E). Positive listing of ingredients, which are reasonably assessed as safe, is the best way to assure safe cosmetics. Such a list excludes the use of compounds outside the list in the formulation of cosmetics. At present, however, integral positive listing of all cosmetic ingredients, is not possible because of the lack of toxicological data. For important ingredients of which the main toxicological

properties are known, provisional lists can be compiled, which should be valid only for a short term, for instance 5 years.

Instead of one large integral positive list the introduction of small classified positive lists will be more practical and surveyable. A model of such small positive lists has been designed in Table 1-X.

(F). Labelling problems must be considered in a projected legislation and should involve the following: defined designations of the product · name; statement of the responsible firm; batch code; net contents and price per unit amount. Disclosure of potentially sensitizing compounds can be considered, which would aid the sensitized consumer in avoiding certain sensitizers.

(G). Efficacy statements can for instance be considered in a cosmetic legislation by a "positive list" of truthful efficacy statements, such as has been mentioned in the previous section. Defined and mandatory designations of a product's name might also prevent exaggerated efficacy suggestions of cosmetic products. An alternative way is to make scientific proof of claimed cosmetic actions which must be assessed by an expert panel. If the special cosmetic action is related to specific expensive ingredients (for instance protein hydrolysates, egg yolk, placenta extracts, vitamin A, royal jelly, avocado oil) quantitative statements must be made mandatory in order to make public control possible. Such statements will satisfy the consumer, but will also protect the bon-afide manufacturer from unfair competition.

(H). A mandatory notification system of composition formulas can be considered. It must be organized in such a way that trade secrecy can be maintained. Such a registration should not be related to safety or efficacy approvals by the government, as is the case with the registration of pharmaceutics and pesticides. The purpose of such a system is to give an efficient surveyability to the government with particular attention to the use of "safe" ingredients. Moreover, such a system creates the immediate availability of the formula to the medical profession in case of adverse skin reactions and accidental poisoning. On occasions where manufacturing errors can create very dangerous situations of massal poisoning, the availibility of the formula will facilitate chemical analytical control by the public health authorities.

1.9. Existing legislations.

No attempt will be made here to present a complete study of existing legislations. Very accurate details of national cosmetic regulations of many countries in the world are compiled by the International Federation of Societies of Cosmetic Chemists (IFSCC, annually since 1970). In this section a small number of existing legislat-

TABLE 1-X POSITIVE LIST OF CLASSIFIED INGREDIENTS.

A model.

(A) COLOURS

		Fields of application and max.% .1	.2	.3	.4	.5	.6	.7	
Red 1	CI 12085	3	-	-	-	-	-	+	
Red 2	CI 45380	+	+	+	-	-	½	+	..Purity requirements.

.1 = lipstick
.2 = mouth,teeth
.3 = eye area
.4 = hair (non-oxydative)
.5 = skin in general (long stay)
.6 = aerosol
.7 = all others

..Label warnings.

..If provisional, state number of years

(B) ANTIMICROBIALS

	Fields of application and maximum % .1	.2	.3	.4	.5	.6	.7	.8	
Formaldehyde	0.2	0.1	-	0.1	-	-	-	5 nail	..Purity requirements.
Paraben-me	0.3	0.2	0.2	-	-	-	-	0.3	
Paraben-pro	0.1	0.05	0.1	-	-	-	-	0.1	..Label warnings.
Hexachlorophene	-	0.05	-	-	0.5	1	-	-	

.1 = preserv.single
.2 = preserv.combin.
.3 = baby skin
.4 = skin,long stay deodorant
.5 = mouth,teeth
.6 = skin,short stay; soap
.7 = aerosol
.8 = others.

NB for deodorant spray lowest of 4 and 7.

..If provisional, state number of years

(C) UV-ABSORBERS

	Field of appl.-max% .1	.2	
2.Ethoxyethyl.p.methoxycinnamate	5	0.1	..Purity requirements.
2.2'.4.4'.tetrahydroxybenzophenone	5	0.2	

.1=sunscreen
.2=colour stabiliser.

..Label warnings.

..If provisional, state number of years.

ions will be discussed to give an idea of the differences in rule making in the different parts of the world.

(USA). The Food Drug and Cosmetic Act of 1938 does not include in its definition products which protect the body or which prevent disease, such as sunburn preventives, deodorant-antiperspirant, antiseptic soaps and fluorinated dentifrices. These products are regarded as drugs, and regulated as "over-the-counter" drugs (Federal Register, 1972) which can be sold freely without prescription. These OTC-drugs have recently been critically examined for their safety, efficacy and accurate labelling (Giovachini, 1975). These criteria have, however, not yet been defined for cosmetics in general. For the present the FDC-act prohibits the trade of "adulterated" and "misbranded" cosmetics. Adulteration is concerned with the cosmetic substance, and has been defined by the law as not containing injurious ingredients, nor being prepared or stored under unsanitary conditions. Misbranding has to do with the label and the package. The law defines misbranding as untruthful, false or misleading information. Unfortunately, the burden of proof for adulteration and misbranding lies with the government, which in many cases is difficult with only such generalized definitions. It is therefore not surprising that the government with such a weak statutory situation has often considered the promulgation of special regulations under the protection of existing laws, to detect adulteration or misbranding without further proof. It could be for instance by the creation of negative, positive or limiting lists of ingredients, such as is the trend in Europe. It has been done, however, in a very restrictive sense. At present the following special regulations exist: a positive list of synthetic organic colours with mandatory certification for each batch which can meet the defined purity standards; negative listing of bithionol, mercury compounds, vinylchloride and hexachlorophene (with minor exceptions). In relation to misbranded cosmetics the following special regulations exist: obligation of a minimum of 2% egg in egg shampoos and more recently a mandatory qualitative formula labelling (Federal Register, 1974). This latter regulation has been promulgated under another law, namely the Fair Packaging and Labeling Act, that is also valid for cosmetics and which states that labelling should facilitate value comparisons. It remains doubtful, however, whether such formula statements would aid the consumer in his purchase. The most recent rule-making regulation under the existing legislation in the USA is the "voluntary cosmetic registration programme" (FDA, 1972), which is now in its fourth year. All batches of produced cosmetics should be voluntarily registered in detail, including raw material composition to a computerized data bank. Also all product experience records of the manufacturers should be voluntarily registered. Computerized data will contribute very useful information in regard to cosmetic safety.

(EEC) . The EEC cosmetic directive that has been accepted in July 1976, has made an approach to cosmetic safety by an enormous negative list (EEC,1976 ; Annexe II)

of more than 400 substances. Many of these substances never have been and probably never will be used in cosmetics. It is doubtful whether such a list has a practical significance, except if it is reduced to a small number of compounds, which have or will have cosmetic interest. More useful is a limiting list (EEC, 1976; Annexe III-1) of dangerous materials, but which are technologically indispensable. These compounds are permitted below defined levels and for defined field(s) of application. This list is a limiting list in the negative sense, because other substances outside the list can still be used. Annexe III-2 of the directive, however, has a positive character, because only these colours can be used for application where there is a possible contact with mucous membranes. Annexe IV is a provisional list valid for 5 years, containing materials of which there is insufficient toxicological information. By these listing systems there is still no guarantee that new injurious ingredients will not be introduced in cosmetics. Because of this danger article 11bis has been added in the directive, which states that within one year after the expiry period for the implementation by the member states appropriate propositions for the establishment of lists of permitted substances (positive lists) should be studied. Regarding formula disclosure, the directive states that in case of accidental poisoning (article 7.3 of the directive) adequate and sufficient information regarding harmful substances should be made available to the competent authority of public health.

(Swiss) . Legislation on cosmetics (Schweiz.Eidg.Gesundheitsamt,1967) has made a start on a toxicological approach towards safety. The LD_{50} (oral toxicity, rats) of cosmetic products which come into contact with the mucous membranes, should be lower than 5 g per kg bodyweight. For all other cosmetics the limit is 0.5 g per kg bodyweight. Swiss legislation also has a positive list of pharmacologically active substances, which means that no other pharmacologically active compounds outside the list can be used. Unfortunately no definition is made for the term "pharmacologically active". Preservatives and colours seem to be regarded as "additives" and thus are outside the scope of the list. The list will be periodically updated to the current state of cosmetic science (Wiesmann, 1975), but the latest version of 1970 (Schweiz.Eidg.Gesundheitsamt, 1970) contains only 3 sunscreens and 17 antimicrobials, which is probably far below the number of actually used compounds in Switzerland. According to an inquiry up to January 1975 in the Netherlands (Head-inspection of Public Health, 1975) 16 sunscreens and 41 antimicrobials are used in cosmetic products in the Netherlands. Regarding qualitative label claims of pharmacologically active substances, an obligation in the law is stated that at least 10% of the defined limit on the list should be present in the product. Vitamin claims must be annually certified by the government. The claim is analytically controlled by a public laboratory and at the expense of the manufacturer. The Swiss legislation also has an indicative list of efficacy statements to

prevent exaggeration and untruthfulness of information to the consumer.
(The Netherlands) . The first Dutch Cosmetic Act (Cosmeticabesluit,1968)has formulated the safety of cosmetics as follows: cosmetics shall never be injurious, nor possibly be injurious, if employed according to their directions(article 2.1). The harmfulness to health has, however, not been defined toxicologically in the act. Besides a negative list of 14 toxic elements and 20 organic ingredients, the act has a limiting list (in negative sense) of 20 ingredients with defined limits of concentration, fields of application and mandatory label warnings. A second version appeared in 1976, in which two positive lists were added, namely of eye make-up colours and of quaternary ammonium compounds for use in mouth, skin, scalp and hair products. Formula disclosure is not mandatory in the Netherlands, but such a submission occurs frequently on the basis of cooperation by manufacturers, in cases of adverse reactions or doubts of safety. A practical version of the Dutch Cosmetic Act 1976 has been published by Liem (1976).

2. Analytical aspects.

The safety of cosmetics can be most precisely judged by toxicological criteria. This matter has been extensively discussed in several sections of the previous chapter. The laboratory procedures for testing toxicological safety, however, are very tedious, time consuming and expensive. For these reasons it is not practical to define such toxicological criteria for the daily and public control of cosmetics.

Most countries have therefore adopted the system of negative and positive listing of ingredients with known toxicity. Such a system can be conveniently controlled by chemical analysis.

Considering the ca. 6000 possible ingredients for cosmetics, one can expect a wide variety of complex formulations, even for one type of product. This fact will certainly increase the difficulties in clean-up procedures needed before the actual determination of a risk-bearing compound can be started. A great aid to the analytical cosmetic chemist would be the knowledge of the product formula. But as this formula is a trade secret and as no obligation of disclosure exists in the Netherlands, the analysis for the daily public control of cosmetics is a difficult task.

2.1. Formal approach of public analytical control of cosmetics.

A formal approach for one sample, to investigate whether the product meets the requirements of an existing legislation, will result in a laborious analytical scheme. An example of such a scheme on behalf of the Dutch Cosmetic Act will be given here.

(a) Limit reactions for 14 toxic elements of the negative list should first be performed. In general pre-mineralization of the sample is necessary. The Dutch Cosmetic Act has stated "absence" of these toxic elements and the Analytical Committee for cosmetics has defined "absence" of a toxic element as "less than 1 µg per g"

(b) Characterization reactions for each of the 18 compounds or groups of compounds on the negative list. Any identification of such a compound must be confirmed, preferably by an analytical method other than that used for the identification.

(c) Characterization reactions for the 23 compounds of the limiting list must also be made. If such a compound is detected, a quantitative determination should be performed, not only to control the concentration level, but also to serve as a confirmation of the detected compound.

(d) If the product is an eye cosmetic, the colour(s) must be identified and the

results checked with the positive list of the Act.

(e) If the product contains a quaternary ammonium compound, identification, confirmation and quantitative analysis must be made and the results checked for the limitations and the positive list of the Act.

Such a complete analysis of one sample will last for several months. The analytical scheme for the EEC-directive is even more laborious. The negative list of the Directive consists of more than 400 compounds and the limiting list of more than 40 compounds. Moreover a huge positive list of approximately 100 colours has also to be checked for coloured products which can come into contact with the mucous membranes.

This formal and systematical approach for the daily public control of cosmetics is therefore not practical. A more practical approach has to be made.

2.2. Practical approach of public analytical control of cosmetics.

The basis for a practical approach of public analytical control of cosmetics is a study of cosmetic technology and formulation from textbooks, scientific papers and patents. From such information an anlytical guide of Table 2-1 can be compiled. In the guide any claimed cosmetic action is related to the probable use of an ingredient from the negative list. With the aid of such an analytical guide the analysis is reduced and restricted to a small number of toxic compounds.

The control of positive lists will not give many problems, providing the number of compounds on the list does not exceed a reasonable number. Up to 20 compounds can be handled properly by reference analysis. An example of an existing system is the paperchromatographic reference analysis of permitted food colours of the Dutch Food and Commodities Law ("Warenwet"). If, however, the number of compounds exceeds 20 - for instance the EEC colour list of approximately 100 compounds - reference analysis will be very laborious.

The Dutch positive list of quaternary ammonium compounds is another example of a positive list that will be difficult to control. The list consists of 22 compounds, but most of the compounds are indicated in terms of generalized radicals, such as alkyl, alkylene, alkoxy, which has been defined as radicals of 8-22 carbon chains. If all the possibilities are considered, the list consists of more than 100 compounds. A reference analytical identification system will not be practical in this case. In all these cases of a large number of possible compounds, the most practical solution is a mandatory notification of the quaternary ammonium compound ingredient, or the colour compound used. The analysis can then be limited to the confirmation of the disclosed compounds.

The availability of reference compounds of sufficient purity is not easy.

TABLE 2-I

ANALYTICAL GUIDE

Dutch Cosmetic Act 1976

Claimed cosmetic action from label or pamphlet information	Ingredients to be analysed
Hair cleaning(shampoos)	Formaldehyde,nitrobenzene
colouring	p.Phenylenediamine, p.toluenediamine, resorcinol, pyrogallol, lead acetate, siver nitrate, complex chrome dyes
bleaching	Hydroxyquinoline
waving	Thioglycollic acid, aniline, dimethylamine, alkaline agents, pH.
waving-neutralizer	Acidic agents.
straightening	Thioglycollic acid, KOH or NaOH, pH.
conditioning	Quats, organo-mercurials.
care	Quats, organo-mercuriàls, triarylphosphates, methanol.
setting	Quats. (for hairspray see also under aerosol).
growth stimulant	Pilocarpine, androgens, oestrogens.
anti-dandruff	Hexachlorophene, quats, selenium sulfide.
Face care-moisturizing	Formaldehyde, vitamin A, vitamin D, sulphonamides, corticosteroids, oestrogens, organo-mercurials, hexachlorophene, resorcinol, antibiotics.
mask	Formaldehyde, oestrogens.
anti-wrinkle	Oestrogens.
bleaching	Monobenzone, mercury.
cleansing	Formaldehyde, methanol, hexachlorphene, antibiotics, quats.
refreshing	Formaldehyde.
shaving aids	Methanol, dichloromethane, hexachlorophene, benzocaine.
make-up	Sr-Ba-Zr colour lakes.
Eye make-up	Sr-Ba-Zr colour lakes, colour, formaldehyde, hexachlorophene, organo-mercurial, b-naphthol.
others	Methanol, formaldehyde, oestrogens, quats, formaldehyde.
Lip make-up, lipsticks	Sr-Ba-Zr colour lakes.
Mouth refreshing	Methanol, boric acid.
dentifrice	Chloroform, vitamin A, Sr chloride, Zr abrasives.
Body/hand cleaning	As under face cleaning.
perfuming	Methanol, boric acid.
moisturizing	As under face moisturizing.
sunburn prevention	Benzocaine, monoglyceryl p.aminobenzoate, isoamyl N.N. dimethyl p.aminobenzoate.
anti-perspirant	Zr compounds, formaldehyde, hexamine.
deodorant	Hexachlorophene, antibiotic, boric acid, formaldehyde, (for deodorant sprays see also under aerosol).
depilation	Sr sulfide, Ba sulfide, Sr thioglycollate, thioglycollic acid, thallium compounds, pH.
Nail softening	KOH or NaOH, pH.
hardening	Formaldehyde.
others	Methanol, chlorinated solvents, triarylphosphates.
Foot preparations	Hexachlorophene, formaldehyde, hexamine, Zr compounds, hydroxyquinoline, boric acid, quats.
Baby preparations	Formaldehyde, hydroxyquinoline, boric acid, quats, organo-mercurials, hexachlorophene.
Feminine hygiene	Antibiotic, hexachlorophene, formaldehyde, (for intimate sprays see under aerosol).
bust improvers	Oestrogens.
Aerosol	Chlorinated solvents, inflammable compounds, dichloromethane, 1.1.1.trichloro-ethane, vinylchloride, methanol

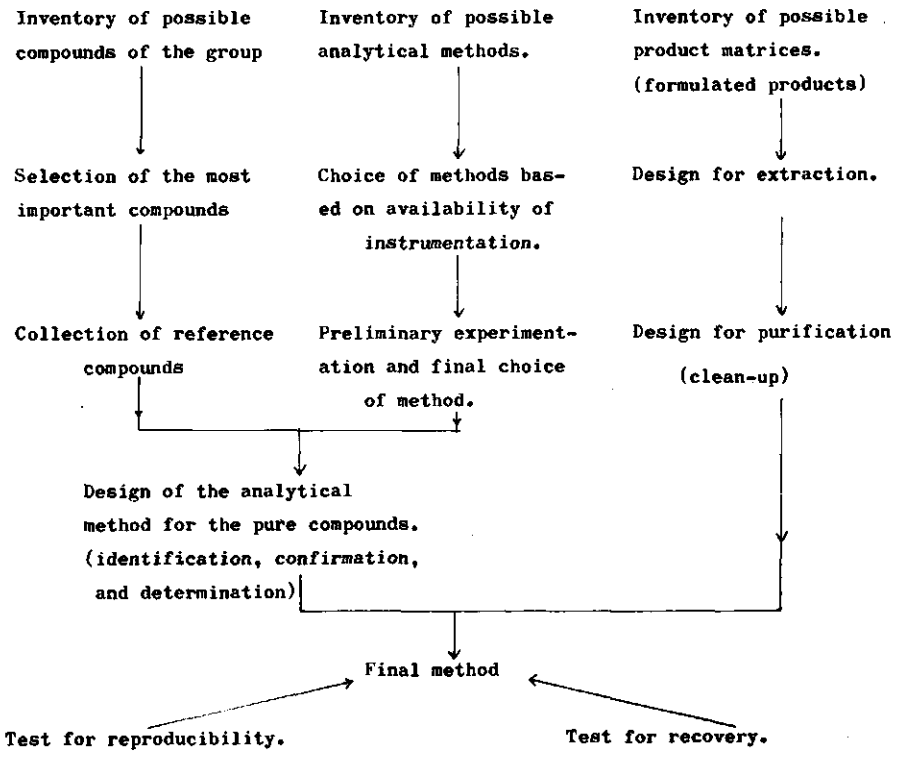
Industrially available ingredients do not always meet the same specifications of composition and purity. A good example is the commercially available xanthene colours for lipsticks (see Figure 3-A), which contained in several instances 6 impurity compounds in appreciable quantities. We consider the organization for the availability of sufficiently purified reference compounds on an international basis as an important project for the public analytical control of cosmetics.

2.3. Blueprint for the analytical approach in this study.

This study has been made to obtain actual information on the use of several classes of risk-bearing compounds in marketed cosmetics. But as many methods of analysis are not publicly available, a sytematic approach has been made for each class of risk-bearing compounds. The basic blueprint for all these studies is described in Table 2-II. It begins with the inventory of possible compounds taken from one class that will be studied. All kinds of information must be gathered, textbook information, scientific publications, patents, technological pamphlets from chemical supply houses etc. It is, however, unlikely that the cosmetic industry has made use of all these compounds in practice. A selection must therefore be made of the most important compounds. For practical reasons approximately 20-25 compounds have always been selected to simplify the analytical study. With the cooperation of the chemical supply houses a collection of the selected reference compounds must be started. This is usually a painstaking and time-consuming business. Meanwhile an inventory of analytical methods must be made from scientific literature sources and a first choice of several methods made, based on the availability of instrumentation. After preliminary experimentation a final choice of the analytical method can be made. The third part of the study is the most important, but usually the most difficult. All possible types of formulated products, in which the compound can be incorporated, must be collected, and the study started with spiked and un-spiked products to design the most efficient extraction and clean-up procedure. The final method of determination can then be set up. The method must then be tested for reproducibility and recovery.

This study has been aimed at building up final methods, that are not only reliable, but also as simple in operation as possible. A good example of such an ideal method is the determination of phenolic antimicrobial compounds in all kinds of cosmetics (see section 3.7.2.) and which consists of the following parts: (a) extraction, just by a "weigh and shake" procedure of the sample with a suitable solvent. (b) a "visible" TLC clean-up procedure on ordinary commercial silicasheets. (c) a gaschromatographic determination with "on-column"derivatization. In other analytical methods the entire clean-up procedure can be omitted. An example of such a method is the determination of formaldehyde (see section 3.7.3) which only consists of a dilution step with water followed a fluorometric or a polarographic determination.

TABLE 2-II BLUEPRINT OF APPROACH FOR THE ANALYTICAL STUDY.



3. Methods and results of analytical market surveys

3.1. Eye make-up colours.

Application of colours in the area of the eye should be carried out with the utmost care and with the best non-toxic colours available, in order to minimize the hazard of damage to this vital organ.

In contrast to lipstick colours no practical data is available on the kinds of pigments that are in actual use in today's eye cosmetics. At the beginning of 1972 an analytical survey of these colours was started. As no analytical methods were available such a system was developed. To get an idea what kind of colours might be used an inventory was compiled which is tabulated in Table 3-I. Many are inorganic insoluble pigments. From this point of view instrumental analysis of the metal elements would give much information. Emission spectroscopy, Röntgen fluorescence and Röntgen diffraction methods are the methods of choice. On the other hand it should also be possible to make an analysis along classical chemical lines. A non-instrumental methodology has been used but excluding the identification of the whites (except for TiO_2). This can only be done satisfactorily by instrumental analysis.

3.1.1. Apparatus and reagents.

Apparatus for TLC	Kjeldahl flasks (25 ml)
Microburner	Microscope
Platinum needle	Vitreosol or quartz crucibles (ca.20 ml)

Homogenizer (for instance Ultra Turrax: TP 18/2, Janke & Kunkel, W.Germany)
Centrifuge

Sulphuric acid conc.	Ammonium molybdate 0.5% in conc.sulph.acid	
Sulphuric acid 4M	Methanol	Petroleumether 40/60
Hydrochloric acid 6M	Benzene	Carbontetrachloride
NaOH 8M	Dimethylformamide	Glycerol
Nitric acid 50%	Borax	Polyamide powder MN-SC6 or similar
Diphenylcarbazide 1% in ethanol	K bisulphate	Pb acetate/cotton plugs
Hydrogen peroxide 3%	K ferrocyanide	
Nessler reagent (K_2HgI_4) for ammonia	Sodium peroxide granules, for instance Merck no 6563	

TABLE 3-I INVENTORY OF EYE MAKE-UP COLOURS. (1972)

(numbers refer to colour index numbers)*

GREEN
77288 Chromic oxide
77289 Chromic oxide hydrate
75810 Copper chlorophyllene
77013 Ultramarine green
— Copper versenate
— Titanated mica (green)
Synthetic organic colours and their lakes.
e.g.
42170
44090

VIOLET
77007 Ultramarine violet
77745 $Mn_3(PO_4)_2.7H_2O$
77742 $Mn(NH_4)P_2O_7$
Synthetic organic colours and their lakes.
e.g.
73385

YELLOW-ORANGE
77489 Iron oxide yellow
75300 Turmeric
75120 Anatto
75130 Carotene
— Titanated mica (gold)
Synthetic organic colours and their lakes.
e.g.
11920
19140

BROWN
77492 Iron oxide brown
— Burnt sienna
Burnt umber
Caramel

PEARL
— Guanine
— Titanated micas, talc
— BiOCl, or precipitated on mica
77480 Gold powder
77820 Silver powder
77400 Copper-bronze powder
77000 Aluminium powder

BLUE
77007 Ultramarine blue
77346 Cobalt aluminate
75510 Ferricferrocyanide
— Titanated mica (blue)
Synthetic organic colours and their lakes.
e.g.
42735
42090
Sudan blue II

RED
75470 Carmine
77491 Iron oxide red
77007 Ultramarine red
— Titanated mica (red)
Synthetic organic colours and their lakes.
e.g.

12120	15850	45430
12150	15865	45170
12085	15880	45425
	15630	

BLACK
77499 Iron oxide black
77267 Bone black
77266 Carbon black
— Vegetable black
Graphite

WHITE
— Talc
77891 TiO
77947 ZnO_2
— Zn carbonate
77005 Kaolin
77002 Aluminum oxide
77120 $BaSO_4$
77713 $MgCO_3$
77220 $CaCO_3$
77231 Gypsum
— SiO_2
Tin oxide
Stearates of Li, Mg, Ca, Al, Zn

* Rowe Colour Index, second ed., Bradford, England (1956).

TABLE 3-II

POSSIBLE GROUPS OF COLOUR-COMPOUNDS FOR THE ASSESSED COLOUR COMPONENTS.

GREEN
Chromium-oxides
Ultramarines
Organic colours

BLUE
Ultramarine
Iron-compounds
Cobalt-compounds
Organic colours

VIOLET
Ultramarines
Manganese-compounds
Organic colours

RED
Iron-compounds
Organic colours

YELLOW-ORANGE
Iron-compounds
Organic colours

BROWN
Iron-compounds
Manganese-compounds

BLACK
Carbon
Iron-compounds

PEARL
Pearlescent compounds

WHITE
White compounds

3.1.2. Methods.

Additives should first be removed from the sample and the pigments isolated. There are three procedures for doing this, which depend on the physico-chemical properties of the sample. In these operations centrifuging is an important step, since the striated sediment will in general give visual information of the separ - ated components.

Hydrophylic samples (miscible with water) : procedure A
Lipophylic samples(immiscible with water) : procedure B
"Wax cake" for instance block mascaras : procedure C

Experimental:

Procedure A. Mix 1 g sample with 40 ml water. Homogenize with blender or Ultra Turrax homogenizer. Centrifuge in 45 ml tube for 15 min. at 3-4000 rev/min.

N.B. The striated sediment will give valuable information, in particular, of which components the pigment mixture consists. The analysis will proceed according to these assessed colour components of the striated sediment. The supernatant liquid might be coloured. If the colour can be absorbed with polyamide powder (procedure under section 3.2.1.), the colour is in its water soluble form. If not the colour might be a suspension of small particles of dye-lakes, which is mainly present in the sediment.

Procedure B. Mix 1 g sample with 20 ml petr.ether. Heat gently on a water bath. to de-fat the powder thoroughly. Decant the petr.ether fraction. Repeat the de-fatting procedure twice more. Dry the powder and suspend in 40 ml water. Proceed as under A.

Procedure C. "Wax cake" mascaras might contain chromic and iron oxides, and the kinds of carbon blacks. For the identification of the carbon types method A or B must be used. For the identification of chrom- ic or iron oxides the sample should be ashed on a platinum dish to remove additives and the carbon.

The next step is to assess the colour components visually by observation of the original sample and of the striated centrifugal sediment. Assessment should be within the following defined colour groups:

Green	Red	Black
Blue	Yellow-Orange	Pearl
Violet	Brown	White

For instance bluish-green should be assessed as Blue and Green. This assess- ment of colour components of the sample is necessary to guide the analysis.

For each of the assessed colour component the possible groups of colour compounds is stated in Table 3-II.

All the possible groups of compounds should now be investigated systematically by the characteristic reactions and methods of section 3.1.3. and that belong to each group of compounds.

3.1.3. Characterization reactions and methods.

1. Ash. Ash a small sample on a platinum dish, heated by a micro-burner. Observe the colour changes during the heating and after cooling.
2. Sulphuric acid 4M . Add a small amount of the isolated pigment to 0.5 ml of the acid. Observe before and after gentle heating. Gas evolution and colour changes might occur. H_2S gas can be smelt and chemically confirmed by the browning of lead acetate/cotton plugs.
3. Hydrochloric 6M. Heat a little of the ash residue (1) with 0.5 ml of the acid. Colouration might occur.
4. NaOH 8M. Cdd a little of the isolated pigment to 0.5 ml of NaOH 8M. Gas evolution or colouration might occur.
5. Aqua regia(Nitric acid 50% + HCl 6M, 3+1 by volume). A little of the isolated pigment is added to 0.5 ml of aqua regia. The pigment might dissolve and the liquid coloured.
6. Dimethylformamide. Add a little of the isolated pigment to 1 ml of dimethylformamide. Colouration of the solvent might occur.
7. Methanol. Add a little of the isolated pigment to 1 ml of methanol. Colouration of the solvent might occur.
8. Sodium peroxide melt. Melt a small amount of the ash residue (1) with several granules of sodium peroxide in a vitreosol or quartz crucible. Observe colour after cooling. If characteristic reactions should be obtained with this melt, all of the excess sodium peroxide should first be decomposed by excessive heating
9. Borax bead. Melt borax powder on a platinum wire loop with a little of the ash residue (1). Observe the colour of the hot bead and after cooling.
10. Dissolution. Heat a small amount of the ash residue (1) with a mixture of ca. 0.25 g $KHSO_4$ and ca. 0.25 ml conc.sulphuric acid in a small kjeldahl flask over a small flame of a micro-burner. Cool and dilute with water.
11. TLC. Details of TLC identification of organic synthetic colours will be given in the lipstick colours section (3.2.2.).
12. Metal analysis. This will not be discussed here.
13. Microscope. Suspend the isolated pigment in glycerol. Observe plate structures and colour interference under microscope. Compare with reference samples.

3.1.4. Observations.

Chromium oxides	1. Ash. Both pigments turn grey during the ashing, which becomes dull-green after cooling.
CI 77288 Cr_2O_3	2. Sulphuric acid 4M. Chromic oxides are insoluble and remain unchanged by this reagent.
CI 77289 $2CrO_3.3H_2O$	4. NaOH 8M. Chromic oxides are insoluble and colour persists in this reagent.
	8. Sodium peroxide melt. The melt is yellow coloured, due to the formation of a chromate. Cool the melt and and dissolve in sulphuric acid 4M, until reaction is acid. Boil the solution untill the excess peroxide is decomposed. Cool and add diphenylcarbazide reagent. A violet colour indicates chrome.
Iron compounds	1. Ash. All iron oxides turn brown-black during the ashing which turn deep-brown after cooling
CI 77510 Ferricferro-cyanide. Blue.	2. Sulphuric acid 4M. This reagent is only of importance to distinguish Prussian blue (Ferric ferrocyanide) and Ultramarine blue. Prussian blue retains its colour, Ultramarine fades.
CI 77489 Yellow iron-oxide.Ochre. $Fe_2O_3.nH_2O$.	3. HCl 6M. Heat the pigments in this reagent until dissolved. The hot solution is yellow. Dilute with 5 x its volume with water. The yellow colour fades. Add several crustals of K ferrocyanide. A blue precipitate indicates iron.
CI 77491 Red iron-oxide.Fe_2O_3	
CI 77492 Brown iron-oxide.Fe_2O_3	
CI 77499 Black iron-oxide.Fe_3O_4	4. NaOH 8M. As reaction no. 2, this reaction will also distinguish Prussian blue and Ultramarine blue. Prussian blue turns red-brown, but Ultramarine blue retains its blue colour with this reagent.
Ultramarines	1. Ash. All ultramarines turn grey during the ashing which always turns blue after cooling.
CI 77007 Ultramarine, blue or green or violet or pink.	2. Sulphuric acid 4M. All ultramarines decompose with dilute acid, even without heating. The residue is white. H_2S gas evolution occurs, which can be smelt and characterized by the browning of lead acetate/cotton plugs.
	4. NaOH 8M. Ultramarines retain their colour in this reagent in contrast to Prussian blue (see under iron-compounds).

Mn-compounds	1. Ash. Both Mn-phosphates turn white after ashing.
CI 77745 Mn-phosphate $Mn_3(PO_4)_2.7H_2O$	Burnt umber, however, retains its colour.
CI 77742 Mn-violet $MnNH_4P_2O_7$	2. Sulphuric acid 4M. This reaction confirms the phosphate. Mix the ash residue (1) with this reagent. Add ammonium molybdate reagent. Heat. A yellow colour indicates phosphate.
CI - Burnt umber, Fe/Mn containing earth.	4. NaOH 8M. This reaction confirms the NH_4-radical. Mix the isolated pigment with the reagent. Heat gently. Ammonia gas will escape, which can be shown by the yellow-brown colouration of a hanging drop of Nessler reagent.
	8. Sodium peroxide melt. The reaction is only of importance of the two violet Mn-compounds. The melt is green. coloured, due to the formed manganate. Destroy excess peroxide by heating. Cool. Dissolve melt in 2 ml water. The colour is still green. Acidify. The colour turns into violet, caused by permanganate.
	12. Metal analysis. If burnt umber is confirmed, metal analysis should be made. The ratio Fe/Mn might give an indication of the umber origin.
Cobalt compounds	1. Ash. Cobalt aluminate is the only eye make-up
CI 77346 Cobalt aluminate. Co O.Al_2O_3. Cobalt blue.	colour that retains its beautiful blue colour during and after the ashing.
	9. Borax bead. The colour of the borax bead is deep blue.
Carbon	1. Ash. The residue after ashing is of importance. Carbon
CI 77266 Carbon black.	black has no residue. Bone black leaves 80% of white
CI 77267 Bone black.	residue. Vegetable black leaves a smaller amount of
CI - Vegetable black	residue.
	2 Sulphuric acid 4M. This reagent is only of importance to confirm the ash residue of bone black, which contains phosphate. A small amount of the ash residue is heated with 0.5 ml of the reagent and a small amount of amm. molybdate reagent. The phosphates give a yellow colouration.
Organic colours	2. Sulphuric acid 4M. Colouring of the dilute acid
CI 75470 Carmine. (red) Al or Ca lake of carminic acid	after heating might show the presence of an orgamic colour.
CI 75810 Cu-chlorophyllene.	6. Dimethylformamide. Colouring of this solvent by a small amount of the isolated pigment might show the
There are many synthetic organic colours possible	presence of an organic colour. Filter. Concentrate by

evaporation (in the hood) on a waterbath and use the solution for TLC. NB. Many lakes are appreciably soluble in dimethylformamide.

7. Methanol. This solvent will dissolve the unsulphonated colours, leaving the lakes undissolved. Concentrate the solution and proceed with TLC for identification.

11. TLC. Use the xtract of (6) and (7) for TLC using the systems of chapter 3.2.2.

Pearl pigments

CI 77000 Aluminum powder

CI 77400 Copper or Bronze powder

CI 77820 Silver powder

CI 77480 Gold powder

CI - BiOCl as such or precipitated on mica.

CI - TiO_2 precipitated on mica or talc.White-Blue-Red-Green-Gold.

CI - Guanin, pearl essence; 2.amino-6.oxypurine.

1. Ash. Aluminum powder burns with flashes, leaving a white oxide. If there is a strong orange colour BiOCl might be present. This compound turns white again after heating for a short time, but if heating is excessive the colour remains yellow after ashing. TiO_2 and ZnO also turns yellow during the ashing, but not so intense as with BiOCl. The yellow colour turns white again after cooling.

2. Sulphuric acid 4M. will dissolve BiOCl. Then add Na_2S. A brown precipitation of Bi_2S_3 occurs. Copper and bronze powder will dissolve with blue colour, which deepens after addition of ammonia.

3. HCl 6M. Titanated gold micas contain a little iron oxide. Heat with a little HCl 6M, dilute with equal volume of water and add several crystals of K ferrocyanide. After some time a green-blue colouration occurs.

4. NaOH 8M. This is an excellent reaction for aluminum powder, which generates hydrogen. This reaction of aluminum with a base is much better than with an acid. Guanin dissolves in NaOH 8M. Titanated mica retains its colour in this reagent.

5. Aqua regia. This is only important to dissolve gold or silver powder.

10. Dissolution. This is the best method to confirm Ti. After cooling, dilute with 5x water and add several drops of H_2O_2 3%. A specific yellow-orange colouration occurs.

12. Metal analysis. This gives information for talc or mica.

13. Microscope.Mica platelets show beautiful inter-

TABLE 3-III ANALYTICAL RESULTS : MARKET SURVEY OF EYE MAKE-UP COLOURS (1972).

There were 33 GREEN eye make-up preparations
- 28 contain chromic oxides
- 7 contain iron oxides
- 2 contain ultramarines
- 3 contain aluminum powder
- 1 contains BiOCl on mica
- 15 contain titanated micas
- 3 contain lakes of organic colours CI 19140, CI 42090

There were 31 BLUE eye make-up preparations
- 14 contain chromic oxides
- 2 contain ferric ferrocyanide
- 27 contain ultramarines
- 2 contain aluminum powder
- 6 contain BiOCl on mica
- 5 contain titanated micas
- 2 contain lakes of organic colours CI 14290, CI 42090

There were 20 VIOLET eye make-up preparations
- 3 contain iron oxides
- 15 contain ultramarines
- 3 contain Mn-violet
- 1 contains aluminum powder
- 3 contain BiOCl on mica
- 7 contain titanated micas
- 2 contain lakes of organic colours CI 42090, CI 75470 (Carmine), CI 45170
- 1 contains unidentified pigments

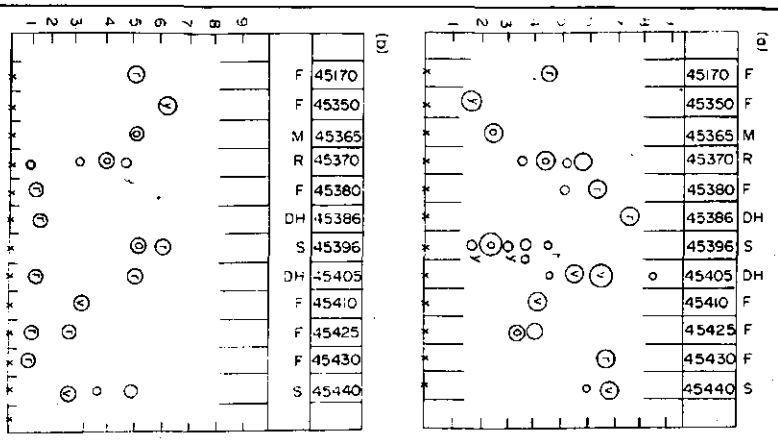
There were 3 RED/BROWN eye make-up preparations
- 2 contain iron oxides
- 3 contain ultramarines
- 2 contain titanated micas
- 1 contains a lake of an organic colour CI 45425

There were 3 BLACK eye make-up preparations
- 1 contains iron oxides
- 3 contain carbon black

There were 5 GREY eye make-up preparations
- 3 contain iron oxides
- 2 contain ultramarines
- 1 contains aluminum powder
- 1 contains BiOCl on mica
- 2 contain carbon black

There were 16 BROWN eye make-up preparations
- 14 contain iron oxides
- 2 contain bronze powder
- 6 contain titanated micas

FIGURE 3-A TLC-PATTERNS OF COMMERCIAL XANTHENE COLOURS



Tlc of reference xanthene colours. All spots are fluorescent under uv light (254 and 360 nm). (a) Tlc system: silica 10 cm precoated plates. Solvent: (vol.) 15 ethylacetate, 6 methanol, 3 conc. ammonia. (b) Tlc system: cellulose 10 cm precoated plates. Solvent: (vol.) 20 Na-citrate (2.5%), 3 methanol, 5 conc. ammonia. Daylight colours: r = red, o = orange, y = yellow, v = violet. Ciphers are Colour Index numbers. Letters: origin of reference substances (lab. or manuf.); F = U.S. Food and Drug Adm. S, DH and M are manufacturers.

ference colours under the microscope. Compare with reference substances.

3.1.5. Analytical results of the "eye make-up colours" market survey.

Sampling from the market was done in January 1972. There were nine brands with 111 samples. All colour tints of three brands were taken. Of the other brands the brown, grey and black colours were omitted, because they were chemically less interesting. All kinds of products were sampled: liquid liner, pencil liner, shadow liquid, shadow compact powder, shadow stick, shadow ointment, mascara wax cake, mascara liquid.

Two unidentifiable colours were found in the 111 samples, namely one blue and one violet. The results are given in Table 3-III.

3.2. Lipstick colours.

Many excellent papers exist on the paper and thin-layer chromatographic identification of lipstick colours (Silk,1965 ; Cotsis & Garey, 1964 ; Perdih, 1969,1972 ; Deshusse & Desbaumes, 1966 ; Unterhalt, 1970; Lehmann et al, 1970). For this reason the choice of methods made for the identification in the 36 samples (18 brands) of lipsticks purchased in February 1972 is only briefly described.

A selection of 40 of the most important lipstick colours was made, based on several recent publications cited above. Reference substances were obtained by the kind cooperation of several manufacturers and laboratories. The list of these selected reference colours is in Table 3-IV.

There are two important groups of lipstick colours worth consideration from an analytical aspect. Xanthene colours are the principle compounds for indelible types of lipstick. It appears that the industrial reference colours in several instances contain minor substances, which are clearly visible on the TLC plates (see Figure 3-A). In the actual separation of the sample extracts, this phenomenon might interfere in the identification of a minor spot. A second important group of colours in lipsticks are the lakes of the water-soluble dyes. Most of these dyes occur as water-insoluble lakes in lipsticks, because of the undesirability of bleeding at the place of application. Most of the lakes are also practically insoluble in other organic solvents, such as ethanol, chloroform or benzene. Fortunately, hot dimethylformamide will dissolve many lakes to an appreciable extent thus permitting extraction for TLC analysis.

TABLE 3-IV. INVENTORY OF THE MOST IMPORTANT LIPSTICK COLOURS (1972)

1	2	3	4	5	6	7	8	9	10	11
12075	—	DC-Or 17	III-b3	Permanent orange	7	–	+	+	–	+
12085	C-Rot 1	DC-Red 36	III-a1	Flaming red	–	+	+	+	–	+
12120	Cex-Rot 1	(DC-Red 35)	IV-a1	Helioechtrot RN	4	–	+	–	–	+
12150	(C-Rot 2)	—	IV-a2	Solvent red 1	–	–	–	–	–	–
13065	Cex-Gelb 10	Ext. DC-Y 1	—	Metanil yellow	–	–	–	–	+	+
14720	C-Rot 54	Ext. DC-R 10	III-a3	Azorubin	–	+	–	–	+	–
15510	Cex-Or 8	Ext. DC-Or 4	III-b6	Orange II	3	+	+	+	–	+
15525	C-Rot 8	—	III-a5	Pigment red 68	–	+	–	–	–	+
15585	Cex-Rot 18	DC-Red 8/9	III-a7	Lake Red C	11	–	+	+	–	+
15630	Cex-Rot 33	DC-R 10/13	III-a8	Litholred lakes	6	–	+	+	–	+
15850	C-Rot 12	DC-Red 6/7	III-a9	Litholrubin B	3	+	+	+	–	+
15880	C-Rot 14	DC-Red 34	III-a11	Deep maroon	6	+	–	+	–	+
15980	C-Or 9	—	III-b7	Orange GGN	8	–	–	–	+	+
15985	C-Or 10	—	III-b8	Sunset yellow	+	–	–	+	–	+
16185	L-Rot 3	FDC-Red 2	III-a12	Amaranth	–	–	–	–	–	+
16255	L-Rot 4	—	III-a13	Cochenille red	–	–	–	–	–	+
19140	C-Gelb 10	FDC-Y 5	III-b9	Tartrazine	8	–	–	+	+	+
26100	—	DC-Red 17	—	Toney red	–	–	+	–	–	+
28440	C-Schw. 6	—	III-d1	Brill. schwarz BN	–	–	–	–	–	–
42051	C-Blau 20	—	III-c1	Patentblau V	–	–	–	–	–	+
42090	—	(FDC-Blue 1)	III-c2	Brill. blue FCF	1	–	+	+	–	+
42735	Cex-Blau 14	—	IV-c5	Acid blue 104	–	–	–	–	–	+
45160	Cex-Rot 26	—	—	Rhodamin 4GD	–	–	–	–	–	–
45170	Cex-Rot 27	—	III-a15	Rhodamin B	1	–	+	+	+	+
45220	Cex-Rot 30	—	—	Acid red 50	–	–	–	–	–	+
45350	Cex-Glb 16	DC-Y 7/8	III-b10	Uranin	–	–	–	–	–	+
45365	(C-Rot 26)	—	—	Dichlorofluoresc	–	–	–	–	–	+
45370	C-Rot 27	DC-Or 5	III-a16	Eosin S 10	16	+	+	+	+	+
45380	C-Rot 30	DC-Red 21/22	III-a17	Eosin Yellowish	17	+	+	+	+	+
45386	C-Rot 31	—	—	Eosin S	–	–	–	–	–	+
45396	C-Or 7		III-b22	Eosin 3G	1	+	–	–	–	+
45400	C-Rot 32		—	Eosin BN	–	–	–	–	–	–
45405	(C-Rot 33)	—	III-a18	Phloxine P	–	–	–	–	–	+
45410	C-Rot 34	DC-Red 27/28	III-a19	Phloxine	2	+	+	+	+	+
45425	C-Rot 35	DC-Or 10	III-a26	Eosin S 15	–	+	–	–	+	+
45430	C-Rot 38	FDC-Red 3	III-a20	Erythrosine	6	+	+	+	+	+
45440	C-Rot 37	—	—	Rose Bengale	–	+	–	–	+	+
73015	—	FDC-Blue 2	III-c6	Indigotine	–	–	–	–	–	–
73360	C-Rot 28	DC-Red 30	III-a21	Helindone Pink	–	–	–	+	–	+
75470	C-Rot 50	—	III-a22	Carmine	–	–	–	–	–	–

1. Colour index number.
2. W. Germany. Mitteilungen III der D. Forschung Gem.
3. USA. Food and Drug Administration Reference.
4. Proposed EEC list for cosmetics (28.12.1971). III = definitely accepted; IV = provisionally accepted.
5. Trivial names.
6. Found in our 36 samples (18 brands).
7. Found by Lehmann 1970.
8. Found by Silk 1965.
9. Found by Cotsis and Garey 1964.
10. Found by Deshusser and Desbaumes 1966.
11. Found by Perdih 1972.

3.2.1. Extraction of lipsticks.

The method as described by Lehmann et al.(1970) was used and which gave five fractions. The (fat)-fraction was discarded; the (dimethylformamide)-fraction and the (lake)-fractions were analysed by TLC ; the (pigment)-fraction was analysed by the methods of the eye make-up colours; if unsulphonated colour compounds are used a (dichloromethane)-fraction is obtained, which can be analysed by TLC.

Experimental: Mix and heat approximately 100 mg of sample with 10 ml dimethylformamide. Filter. Filtrate = (a) and residue = (b). De-fat(a) by shaking with 2 x 15 ml n-hexane. Separate layers. Hexane-layer = (c) and dimethylformamide-layer = (d). This n-hexane layer is the (fat)-fraction.
To (d) an equal volume of water is added. Purify the colours of this fraction by adsorbing the colours on polyamide powder ca. 1 g (for instance type MN-SC6 of Machery and Nagel). Wash with 2 x 10 ml water and 2 x 10 ml methanol in a micro-column, as described by Lehmann et al.(1970). Elute colours with 25 ml of a mixture (methanol-conc.ammonia, 95-5 by volume). This is the (dimethylformamide)-fraction, which is the main fraction of the colours of the lipstick. Concentrate and use the extract for TLC.
In some instances the polyamide powder might still be coloured. Elute with 25 ml dichlormethane. Concentrate. This is the (dichloromethane)-fraction, which contains unsulphonated colours.
The residue (b) contains residual lakes and pigments. Heat with 25 ml of a mixture (methanol-conc.ammonia, 95-5 by volume). Filter and concentrate the filtrate. This is the (lake)-fraction.
The residue still contains a part of the lakes and also inorganic pigments. This residue is ashed to remove organic matter. The (pigment)-fraction is then obtained.

3.2.2. Analytical scheme for the extracted fractions.

(Fat)-fraction	Discard.
(Dimethylformamide)-fraction	Apply TLC with the systems a and b of Figure 3-B The xanthene colours 45370 and 45380 are well identified. The yellow colour 19140 and the blue 42090 should be confirmed by the system c of Figure 3-B. The difficult separation of Orange GGN (15980) and Sunset yellow (15985) can only be done by system d of Figure 3-B. The other orange colours are well identified by the systems a and b of the same figure.

FIGURE 3-B

RF-SCHEME OF LIPSTICK COLOURS.

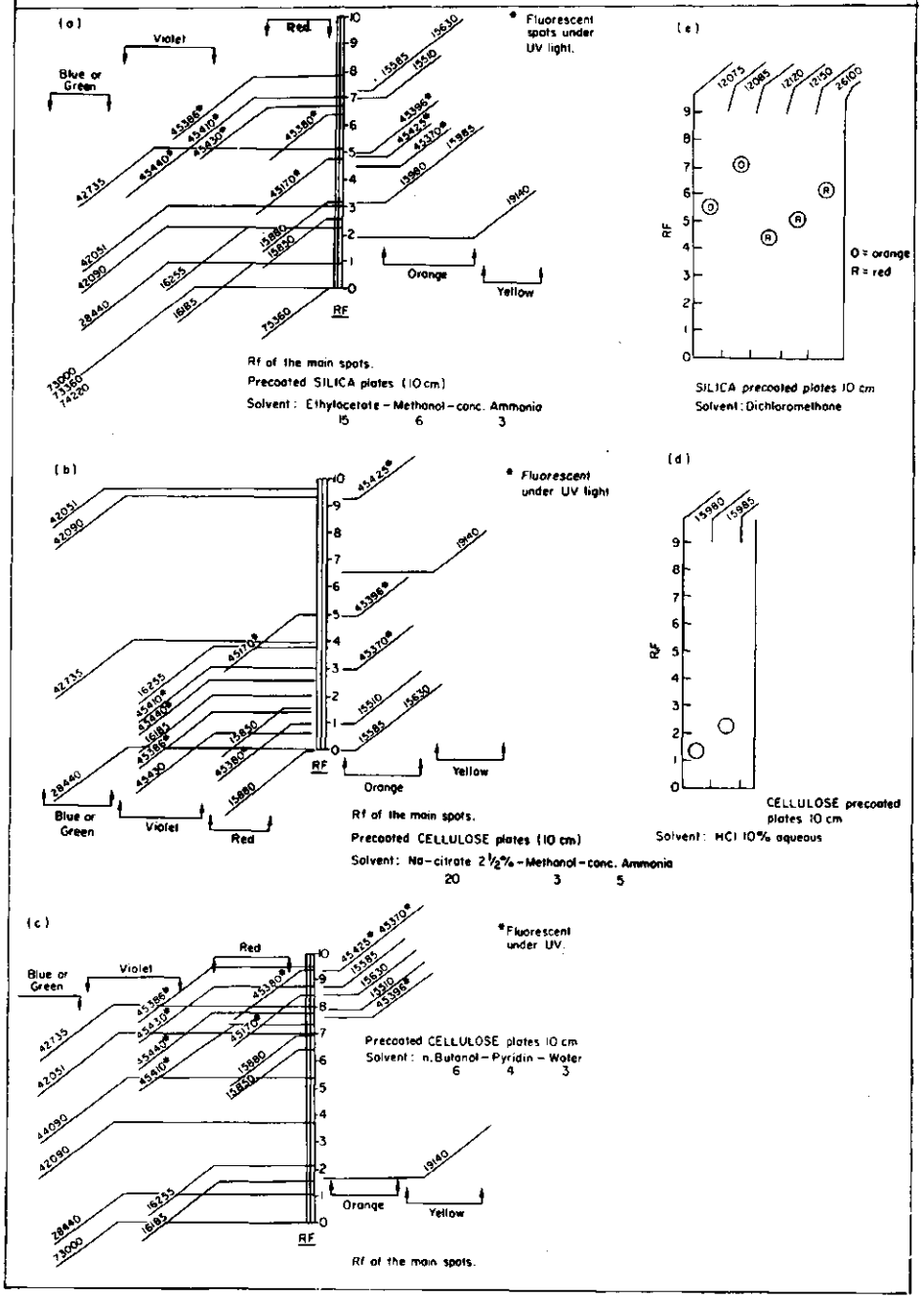


TABLE 3-V ANALYTICAL RESULTS: MARKET SURVEY OF LIPSTICK COLOURS (1972)

Sample brand numbers (two samples per brand)

Colour index numbers	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15		16		17		18		19	
12075										*											*	*			*				*	*					*			
12120			*												*																	*			*			
15510																			*														*	*				
15585		*	*			*	*	*								*				*	*										*	*		*				
15630	*				*																				*				*	*			*					
15850															*							*					*											
15880			*		*								*			*													*	*								
15980											*	*						*					*			*									*	*	*	
19140			*						*		*	*				*		*															*	*				
42090																																		*				
45170										*																												
45370		*	*	*		*	*		*	*			*		*	*			*		*						*					*	*			*		
45380		*	*	*	*		*	*		*			*	*		*			*	*			*		*	*					*		*					
45396																											*											
45410					*															*																		
45430							*		*								*						*			*									*			
Fe-oxide	*	*				*	*		*							*	*	*				*			*	*	*				*							
Ti-oxide	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

(Dichloromethane)-fraction	The unsulphonated colours are identified with the TLC system e of Figure 3-B.
(Lake)-fraction	As under (dimethylformamide)-fraction.
(Pigment)-fraction	Identify by the system as described under eye make-up colours. In most instances TiO_2, titanated micas and iron oxides will be present.

NB. Confirmation of the identified colours should always be made by TLC of mixed spots, namely the sample extract + reference standard.

3.2.3. Analytical results of the "lipstick colours" market survey.

The results of the analytical market survey for 38 samples (19 brands) in 1972 are given in Table 3-V.

3.3. Oxydative hair colour intermediates.

It is well known that the oxidative hair colour intermediates have a significant potential toxicity (Ames et al,1975 ; Searle et al, 1975). More than 100 possible compounds are mentioned in literature (Zviak & Sidi, 1966 ; Tucker, 1967 and 1968). Most of them are substituted derivatives of benzene, naphthalene or pyridine. The most important possibilities are mentioned in Table 3-VI. It is unlikely, however, that all of them are used in modern hair colour preparations. To get an idea of the compounds most frequently in use, 80 samples of 10 brands of the hair colourants for home use as well as for professional use were examined. Most were creams in tubes and some of them were viscous liquids.

Twenty-five reference compounds were selected based on the most recent papers on this subject. They are stated in Table 3-VII. The second step was to build up an analytical scheme, based on a number of excellent papers (Kotteman, 1966 ; Vogel et al.,1968 ; Goldstein et al., 1968 ; Urquizo, 1969). The final choice was made after careful preliminary experimentation.

3.3.1. Analytical scheme for the identification.

TLC for main identification	Preliminary TLC for the estimation of the concentration levels on 10 cm precoated plates, one-dimensional (20 minutes) . Two-dimensional TLC for main identification (2 ½ hours).

GLC aromatic amines	For confirmation and quantitative estimation. No clean-up of extract necessary, because of selectivity of the column. Separation of OFD . MFD . PFD . 26DAP . 34TDA . 25TDA . 24TDA . 2CPFD . 24DAA . Polyphenols and aminophenols do not respond. Quantitative estimation possible.
GLC polyphenols	For confirmation and quantitative estimation. Special clean-up of extract necessary. Removal of amines, aminophenols. Silylation of the phenols necessary. Separation of PCT . RES .HCH . PGL . PHL . AN . BN. (CRES and HCH can be separated at a lower column temperature). Quantitative estimation possible.

3.3.2. Thin-layer chromatography.

Preliminary TLC. The determination starts with a quick (20 minutes) estimation of the concentration levels by TLC on 10 cm precoated silica plates and visualization by iodine vapor. This makes it possible to determine optimum amounts for the spots of the main identification procedure

Two-dimensional TLC on 20 x 20 cm silicaplates was chosen for the main identification procedure. By this technique crude extracts were "cleaned-up" in the first direction, thus permitting excellent resolution in the second. The spots were easily visualized by iodine vapor, which coloured all the classes of intermediates, aromatic amines, aminophenols as well as the polyphenols.

Silica support. There is one important factor worth mentioning. The silica support must be carefully considered to obtain a good resolution and good shapes of the spots. I did not have satisfaction with several kinds of pre-coated supports and also not with self-coated plates of silica with gypsum binder. The purest kind of silica (without binder), for instance Silica HR of Merck, gave the best results. Colouring of the spots by iodine vapour on these kind of self-made plates also gave much more beautiful colours - in particular the starting colours- which aided the identification procedure. On the other hand pre-coated plates only gave brown colours for all the components.

Extracting solvent. A mixture of methanol and ethylacetate (1 + 4, by volume) is always used for sample extraction and preparation of reference solutions, if not otherwise stated. All solutions should always be preserved with several crystals of sodium metabisulphite.

Reference solutions. Make 0.1% standard solutions of the selected reference compounds on Table 3-VII with the recommended solvent mixture. A standard mixture is made by mixing equal volumes of the standard solutions of PFD . 2AF . 3AF . 4AF . RES . AN .

TABLE 3-VI

INVENTORY OF THE MOST IMPORTANT TYPES OF OXIDATIVE HAIR COLOUR INTERMEDIATES

Substituents	No. of isomers	Position	Base	Abbreviation
Diamino	3	ortho	phenylenediamine	OFD
		meta	phenylenediamine	MFD
		para	phenylenediamine	PFD
Dihydroxy	3	ortho	dihydroxybenzene = pyrocatechol	PCT
		meta	dihydroxybenzene = resorcinol	RES
		para	dihydroxybenzene = hydroquinone	HCH
Trihydroxy	3	1.2.3	trihydroxybenzene = pyrogallol	PGL
		1.2.4	trihydroxybenzene = hydroxyhydroquinone	
		1.3.5	trihydroxybenzene = phloroglucine	PHL
Hydroxy amino	3	2.	aminophenol	2AF
		3.	aminophenol	3AF
		4.	aminophenol	4AF
Methyl diamino	6	2.3	toluylenediamine	
		2.4	toluylenediamine	24TDA
		2.5	toluylenediamine	25TDA
		2.6	toluylenediamine	
		3.4	toluylenediamine	
		3.5	toluylenediamine	
Hydroxy diamino	6	2.3	diaminophenol	
		2.4	diaminophenol = amidol base	24DAF
		2.5	diaminophenol	
		2.6	diaminophenol	
		3.4	diaminophenol	
		3.5	diaminophenol	
Diamino methyl ether	6	2.3	diaminoanisole	
		2.4	diaminoanisole	24DAA
		2.5	diaminoanisole	
		2.6	diaminoanisole	
		3.4	diaminoanisole	
		3.5	diaminoanisole	
Nitro diamine	6	3 nitro	1.2.phenylenediamine	
		4 nitro	1.2.phenylenediamine	4NOFD
		2 nitro	1.3.phenylenediamine	
		4 nitro	1.3.phenylenediamine	
		5 nitro	1.3.phenylenediamine	
		2 nitro	1.4.phenylenediamine	2NPFD
Hydroxy	2	α	naphthol	AN
		β	naphthol	BN
Diamino	6	2.3	diaminopyridine	
		2.4	diaminopyridine	
		2.5	diaminopyridine	
		2.6	diaminopyridine	26DAP
		3.4–3.5	diaminopyridine	

TABLE 3-VII

LIST OF REFERENCE COMPOUNDS OF OXYDATIVE HAIR COLOUR INTERMEDIATES.

OFD	o.Phenylenediamine	2AF	2.aminophenol	PCT	pyrocatechol
MFD	m.Phenylenediamine	3AF	3.aminophenol	RES	resorcinol
PFD	p.Phenylenediamine	4AF	4.aminophenol	HCH	hydroquinone
24TDA	2.4.tolylenediamine	M4AF	4.methylaminophenol (metol base)	PHL	phloroglucine
25TDA	2.5.tolylenediamine			PGL	pyrogallol
24DAF	2.4.diaminophenol (amidol base)	2NPFD	2.nitro.p.phenylene-diamine	AN	naphthol α
				BN	naphthol β
24DAA	2.4.diaminoanisole	4NOFD	4.nitro.o.phenylene-diamine	CRES	chlororesorcinol
26DAP	2.6.diaminopyridine				
2CPFD	2.chloro.p.phenylene-diamine				

Sample extraction. Mix 1 g of the product and 4 ml of the recommended solvent mixture. Heat a little on a waterbath. Add ca. 250 mg sodium metabisulphite crystals and ca. 500 mg anh. sodium sulphate. Shake the mixture vigorously. Use the clear liquid for TLC. This extract can only be used for one day !

TLC spotting. Optimal spotting is important. For the 20 x 20 cm plates 5 µg is optimal, except for 2NPFD ($\frac{1}{2}$ µg) and for 3AF . 4NOFD . HCH .AN . BN (10 µg), and for PGL (15 µg). Use half of these amounts for the small (10 cm) plates. Mixed spots of extracts and reference solutions must be applied for confirmation.

Solvent mixtures for TLC. First direction: acetone-chloroform-toluene (35 - 25 - 40, by volume), use unsaturated tank. Develop for approximately 1 hour. Dry for 15 minutes and proceed chromatographing in the second direction with: chloroform - acetic acid - water (50 - 45 - 5, by volume), use saturated tank. Develop for 1 hour and 15 minutes. Dry plate for 10 minutes at 50°C until smell of acetic acid has disappeared !

Visualization. Put severaliodine crystals in an empty chromatographic tank. Place developed TLC plates in this tank. Observe carefully the starting and the definite colours. Take plates out after $\frac{1}{2}$ hour. Excess iodine will disappear, leaving the definite colours of the spots, which is described in the following:

PFD	starts green, change to violet-brown	RES	grey-brown
MFD	yellow-brown	HCH	yellow
25TDA	starts green, change to violet-brown	PGL	brown
24TDA	yellow-brown	2NPFD	red
24DAF	violet-brown	4NOFD	orange
24DAA	yellow-brown	2AF	yellow-brown
M4AF	violet-brown	3AF	brown
AN	violet	4AF	violet-red.
BN	yellow		

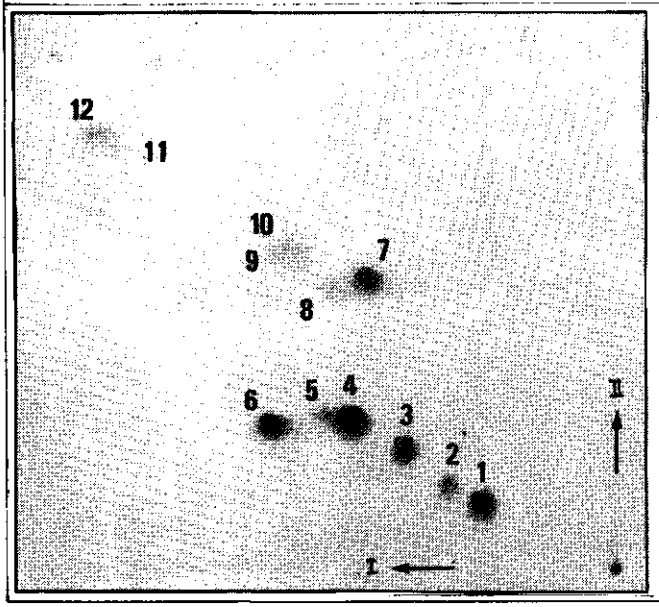
NB. These colours can only be obtained with the purest kind of silica support without binder (for instance Silica HR of Merck). Silica with gypsum binder and commercial precoated silicasheets gave only yellowbrown colours for all these compounds.

Identification. The spots on the two-dimensional TLC plate are identified by relative positions to the standard mixture pattern. An example is shown on Figure 3-C

3.3.3. Gaschromatography of the aromatic amines.

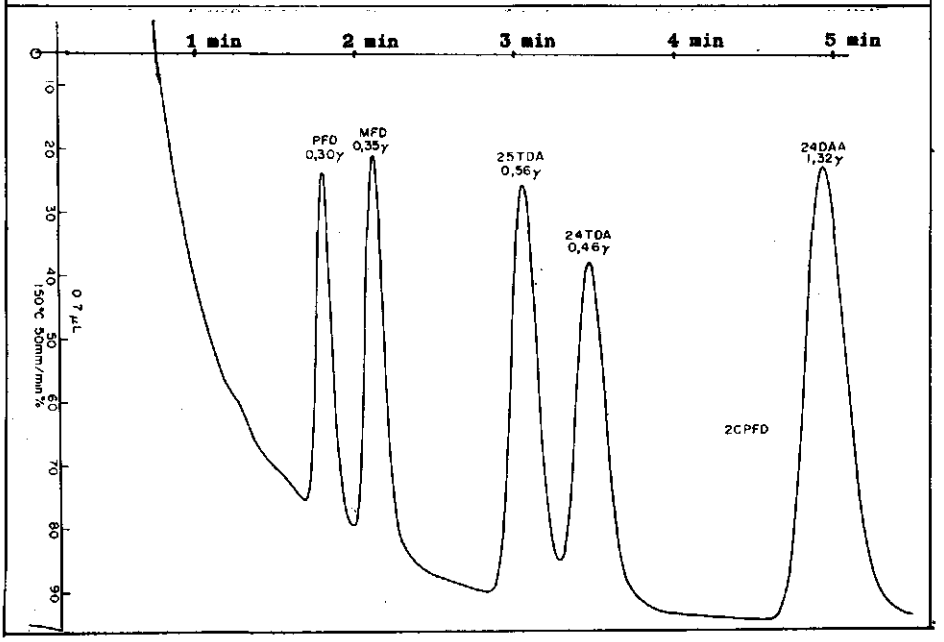
Identification by TLC might still leave doubts, in particular for the difficult pairs RES - HCH , 25TDA - 2.4DAA - MFD , 24TDA - 4AF . Confirmation of the identity of the components by GLC of the important aromatic

FIGURE 3-C. TWO-DIMENSIONAL TLC RESOLUTION OF A REFERENCE MIXTURE.



1 = PFD
2 = 25TDA
3 = 4AF
4 = 3AF
5 = 2NPFD
6 = 2AF
7 = PGL
8 = 2NOFD
9 = HCH
10 = RES
11 = bN
12 = aN

FIGURE 3-D RESOLUTION OF AROMATIC DI-AMINES BY DIRECT GLC.



diamines and separately of the polyphenol group was then obtained.

The aromatic diamines are seperated on an alkaline column (Apiezon L - KOH). Such alkaline columns has also been used by Goldstein et al.(1968), Willeboordse et al.(1968) and Boufford (1968). The aminophenols and the polyphenols do not give response on this column, so that a good confirmation of the identified aromatic diamines by injecting a freshly made crude extract. After several injections, however, the column should be cleaned at a higher temperature, in order to avoid the appearance of "ghost" peaks which generally belong to former injections. The following injection-sequence scheme during the day is highly recommended. Quantitative work is also possible with this column. As an internal standard 2CPFD (2.chloro.para phenylenediamine) can be used..

Injection-sequence: Injection of the GLC standard mixture - exactly 10 minutes
Injection of extract of first sample - exactly 10 minutes
Injection of extract of second sample - exactly 10 minutes
Injection of extract of third sample - exactly 10 minutes.
Cleaning the column at 250°C during 30 minutes

GLC conditions. Column 150 cm X 0.25 inch cuts.diam.,stainless steel. Filling: Apiezon L 6% and KOH 10% on Chromosorb WHP 80/100, which is prepared as follows. Dissolve 700 mg KOH in a small amount of water and then add much ethanol. Dissolve 420 mg Apiezon L in some toluene. Mix both solutions and add to 5.880 g Chromosorb WHP 80/100. Dry the mixture in a rotating vacuum evaporator, keeping the mixture as granular as possible. Column temperature 150°C isotherm. Carrier gas: nitrogen. Detector FID.

GLC standard mixture: Dissolve in 100 ml ethylacetate 45 mg PFD - 50 mg MFD - 80 mg 25TDA - 70 mg 24TDA - 190 mg 24DAA. A chromatogram of this mixture (0.7 µl) is shown in Figure 3-D.

Extraction of samples. The same extraction procedure as for TLC can be used, except that ethylacetate is used as extracting solvent. Only freshly prepared extracts should be used for GLC.

Quantitative determination of 25TDA. The method for 25TDA can easily be modified for the other aromatic diamines. Prepare a solution of the internal standard in ethylacetate (0.1% 2CPFD). Prepare a reference standard solution of 25TDA in ethylacetate (0.15%). With these two solutions the following standard series can be made:

(internal standard)	+ (25TDA)	+ (ethylacetate)	= µg 25TDA per µl
5 ml	1 ml	4 ml	0.150
5 ml	2 ml	3 ml	0.300
5 ml	3 ml	2 ml	0.450
5 ml	4 ml	1 ml	0.600

The standard curve of Figure 3-E was obtained with this standard series of 25TDA solutions. To determine 25TDA in a sample, weigh (m) g of the sample, which must contain 1-6 mg of 25TDA. Mix with 5 ml of the internal standard solution and add

FIGURE 3-E ESTIMATION OF 2,5.TOLUENE DIAMINE BY GLC.

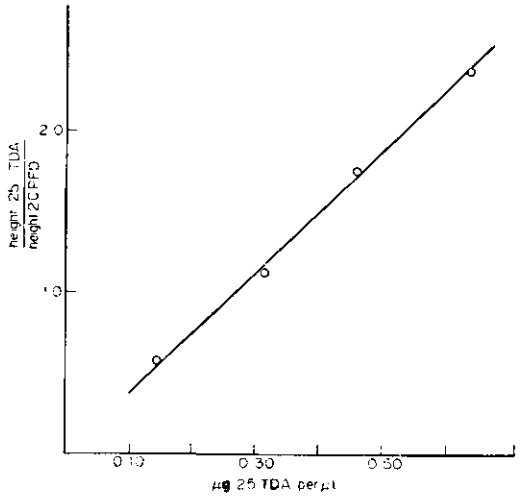
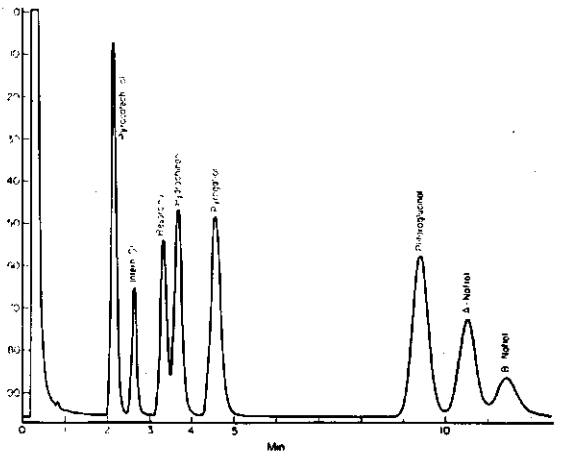


FIGURE 3-F GLC SEPARATION OF SILYLATED POLYPHENOLS.



sufficient ethylacetate to a volume of exactly 10 ml. Homogenize if necessary by heating on a waterbath. Add ca. 1 g anh.sodium sulphate and mix vigorously. Use this extract within 1 hour ! Carry out the GLC according to the projected injection sequence. Calculate the peak height ratio 25TDA:2CPFD. Find the equivalent (a) µg 25TDA from the standard curve. The % 25TDA = $\left(\frac{a}{m}\right)$. From the data of the standard curve of Figure 3-E a variation coëfficient of 1.3% has been found for the standard solutions

(P)=peak height 25TDA:peak height 2CPFD	0.61	1.18	1.81	2.41
(Q)= µg 25TDA per µl	0.156	0.312	0.468	0.624
(P:Q)	3.88	3.78	3.87	3.87

Mean (P:Q) 3.85

Variation coëfficient 1.3%

The accuracy of this method for uncleaned extracts, however, is not better than 5%, due to the retardance of the solvent peak to the baseline, which is caused by the impurities in the extract.

3.3.4. Gaschromatography of the phenolic compounds.

In contrast to the aromatic amines, which can be determined by GLC from crude extracts and without derivatization, the polyphenols in hair colourants can be only determined from purified extracts and after silylation.

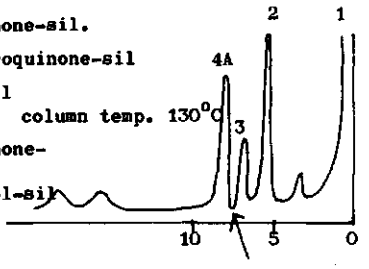
Purification procedure: Shake in a 250 ml separatory funnel the following mixture (4 g sample + 6 ml NaOH 1M + ca. 500 mg Na metabisulphite or Na ascorbate to prevent oxidation + 100 ml dichloromethane). Shake vigorously and wait at least 30 minutes for the separation of the phases. Discard the dichloromethane fraction. Acidify the aqueous fraction with 0.6 ml HCl 35%. Repeat extraction with succesively 50 ml, 25ml and 25 ml dichloromethane. Dry the combined dichloromethane fractions with anh.sodium sulphate. Evaporate the clear extract on a waterbath until dry.

Silylation procedure: proceed with the silylation procedure in a small (ca. 5 to 10 ml) vial which can be closed tightly. Dissolve the dried residue of the extract in 4 ml ethylacetate and add succesively 0.2 ml hexamethyldisilazane (HMDS) and 0.1 ml trimethylchlorosilane (TMCS). Close the vessel and heat on a waterbath of 60°C for 5 min. Cool. The mixture is ready for GLC.

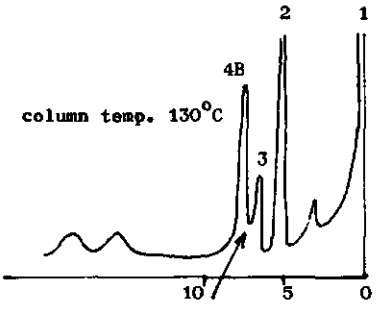
GLC conditions. Column 150 cm X 0.25 inch cuts.diam., glass. Filling: Trifluoropropyl methylsilicone (OV-210) 10% on Chromosorb WHP 80/100. Column temperature 130°C isotherm. FID detector. Temp.inj.port 210°C. Carrier gas: nitrogen. Example of retention times: pyrocatechol 2.3 min. ; nitrobenzene (internal standard) 2.9 min.; resorcinol 3.7 min.; hydroquinone 4.1 min.; pyrogallol 5.1 min.; phloroglucine 10.7 min.; a-naphtol 12 min.; b-naphtol 13 min. An example of a chromatogramme is given in Figure 3-F.

FIGURE 3-G THE PROBLEM OF THE QUASI-HYDROQUINONE PEAK OF SAMPLE no 25.

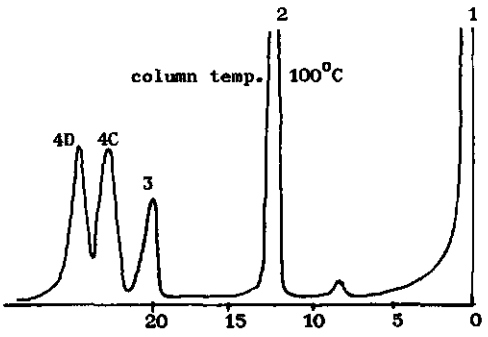
1 = ethylacetate.
2 = nitrobenzene.
3 = resorcinol-sil
4A = quasi hydroquinone-sil.
4B = (4A)+extra hydroquinone-sil
4C = hydroquinone-sil
4D = identified quasi-hydroquinone-peak : chlororesorcinol-sil



Original sample extract
Note the un-common base-line touch between peak 3 and 4



Original sample extract + extra hydroquinone-sil.
Note the common base-line touch between peak 3 and 4



Original sample extract + extra hydroquinone-sil.
Note clear separation of 3 - 4C - D

TABLE 3-VIII ANALYTICAL RESULTS OF THE OXYDATIVE HAIR COLOUR MARKET SURVEY. 1971

Total 80 samples (10 brands)

Brand A (12 samples)
- 11 with 25TDA
- 12 with 3AF
- 12 with RES
- 2 with 2NPFD
- 1 with 4NOFD

Brand B (9 samples)
- 7 with 25TDA
- 4 with 3AF
- 5 with RES
- 4 with 2NPFD

Brand C (5 samples)
- 4 with 25TDA
- 3 with 3AF
- 3 with RES
- 1 with 2NPFD

Brand D (7 samples):
- 5 with 25TDA
- 2 with 3AF
- 3 with RES
- 3 with 2NPFD

Brand E (8 samples):
- 5 with 25TDA
- 2 with 2AF
- 3 with 4AF
- 3 with 2NPFD
- 8 with RES

Brand F (11 samples):
- 10 with 25TDA
- 1 with 2AF
- 3 with 3AF
- 4 with 4AF
- 5 with RES
- 1 with AN

Brand G (6 samples):
- 6 with 25TDA
- 1 with 2AF
- 5 with 3AF
- 4 with 4AF
- 1 with 2NPFD
- 1 with AN
- 2 with 24DAA

Brand H (12 samples):
- 6 with 25TDA
- 6 with 2NPFD
- 6 with RES
- 1 with HCH
- 2 with AN
- 1 with CRES

Brand I (9 samples):
- 7 with 25TDA
- 7 with 3AF
- 1 with 2NPFD
- 7 with RES

Brand J (12 samples):
- 2 with 25TDA
- 2 with 3AF
- 2 with HCH
- 1 with AN

Unidentified spots: 8

Conclusions: No p.phenylenediamine was found.
2.5. Toluylenediamine and resorcinol were the most important compounds.
The 3 isomeric aminophenols are frequently used.
2.Nitro.p.phenylenediamine is much used, 4.nitro.o.phenylenediamine less.
Of minor importance are: α-naphthol, hydroquinone, chlororesorcinol, 2.4.diaminoanisol.

Abbreviations:
- 25TDA = 2.5.toluylenediamine
- 2AF = 2.aminophenol
- 3AF = 3.aminophenol
- 4AF = 4.aminophenol
- 2NPFD = 2.nitro.p.phenylenediamine
- 4NOFD = 4.nitro.o.phenylenediamine
- RES = resorcinol
- CRES = chlororesorcinol
- HCH = hydroquinone
- AN = α-naphthol
- 24DAA = 2.4.diaminoanisole.

Quantitative determinations. In quantitative determinations of resorcinol and hydroquinone, nitrobenzene is used as an internal standard. To plot a standard curve the following standard series are silylated. Prepare 4 mixtures by addition of succesively 2 ml ethylacetate solutions containing 0.6 - 0.9 - 1.2 - 1.5 mg resorcinol or hydroquinone to 2 ml ethylacetate solution (0.25%) of nitrobenzene. Silylate these standard solutions as described before and use 1-2 μl for GLC injection. Calculate the peak height ratio resorcinol:nitrobenzene or hydroquinone:nitrobenzene and plot these data against mg resorcinol or hydroquinone of the standard series, to obtain the standard curve. As the sample weight is known, the % resorcinol or hydroquinone can be calculated. Recoveries of 94% of added resorcinol in practical samples was achieved.

Separation of chlororesorcinol and hydroquinone. In one of the samples a peak appeared on the hydroquinone place and after resorcinol. The separation seemed to be better than usual, namely that the recorder reached the baseline, which was not the case in the standard chromatograms. When the column temperature was lowered from 130°C to 100°C, and a mixture of hydroquinone-sil standard + silylated extract was injected, three peaks were clearly recorded and separated, namely resorcinol-sil , hydroquinone-sil and the quasi hydroquinone-sil peak. Further trials with reference compounds and also TLC information led to the identification of 2.chlororesorcinol for the quasi-hydroquinone. See Figure 3-G

3.3.5. Analytical results of the "oxidative hair colours" market survey.

The market survey was done with 80 market samples in 1971. The results are given in Table 3-VIII. Surprisingly p.phenylenediamine was absent. The most important compounds in oxydative hair colourants are still 2.5.toluenediamine, the three isomeric aminophenols, resorcinol, a-naphthol and 2.nitro.p.phenylenediamine.

3.4. Suntan preparations.

Suntan preparations may contain several classes of potentially risk-bearing compounds, such as sunscreens (UV-absorbers), browning agents (mainly dihydroxyacetone) and local anesthesics (e.g. ethyl.p.aminobenzoate , lidocaine). These cosmetics are for several weeks a year applied intensively on to large surfaces of the body, by old and young alike and at the same time the skin is exposed to sun, air and water. It is possible that under such rigorous conditions of use the toxicological hazards of the active components will increase. To get some idea of the diversity of sunscreens, analytical data of some 66 suntan prepar-

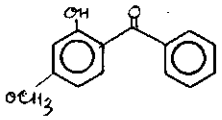
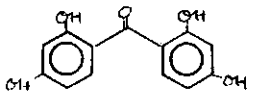
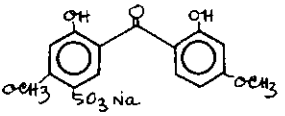
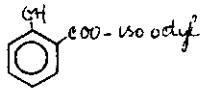
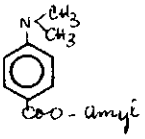
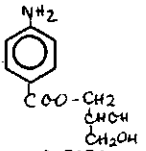
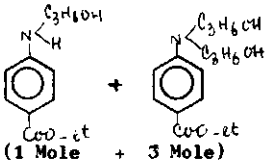
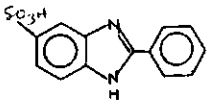
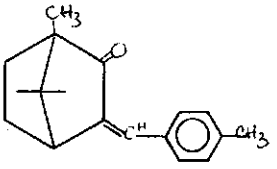
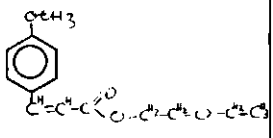
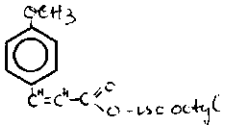
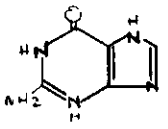
TABLE 3-IX **INVENTORY OF SUNSCREENS.**

A. Para amino benzoic acid and esters: ethyl, butyl, propyl, glyceryl.
B. N.N.Dimethyl para amino benzoic acid ester: isoamyl.
C. Ortho aminobenzoic esters (anthranilic acid esters): methyl, menthyl, phenyl, benzyl, linalyl, cyclohexenyl, bornyl, isobornyl.
D. Salicylic acid esters: menthyl, homomenthyl, phenyl, benzyl, glyceryl, dipropylene glycol, 2.ethylhexyl.
E. Cinnamic acid esters and derivatives: 2.ethylhexyl p.methoxy cinnamate, benzyl cinnamate, 2.ethoxy ethyl p.methoxy cinnamate, menthyl cinnamate, benzyl p.methoxycinnamate, i.butyl salicyl cinnamate, propyl p.methoxycinnamate, butyl cinnamoyl pyruvate, α-phenyl cinnamonitrile, 2.ethylhexyl 2^1.cyano 3.3^1.diphenylacrylate.
F. Dihydroxycinnamic derivatives: umbelliferone, methylumbelliferone, methyl aceto-umbelliferone.
G. Trihydroxycinnamic acid derivatives: esculetin, β.methyl esculetin, dafnetin, glycosides of esculin and dafnin.
H. Benzophenone derivatives: 2.4^1.dihydroxybenzophenone, 4.phenylbenzophenone, carbonic acid isooctylester.
I. Quinine oleate, tannate, stearate, bisulphate.
J. Coumarin derivatives: 7.hydroxy-, 7.methyl-, 3.phenyl.
K. Digalloyltrioleate.
L. Dibenzalazine.
M. Dibenzalacetophenone, benzalacetone.
N. Benzimidazolen, phenylbenzimidazolon sulphonic acid Na.
O. 2.Phenyl benzoxazole, methylnaphthoxazole.
P. 2.Phenyl benzothiazole.
Q. Stilbene.
R. 2.Acetyl bromo indazole.
S. 8.Hydroxy quinoline, 2.phenyl quinoline.
T. Butyl carbityl 6^1.propyl piperonyl ether.

TABLE 3-X **LIST OF INDUSTRIAL SUNCREENS FOR REFERENCE.**

Firmenich	Solprotex 1, 2, 3	Salicylates.
Givaudan	Parsol mcx	2.ethylhexyl p.methoxycinnamate.
	Parsol ultra	Mixture, not known.
	Givtan F	2.ethoxyethyl p.methoxycinnamate.
Laserson Sabety	Ecranosol	Salicylates.
	Solecran	Unknown.
Felton	Sunarome	2.ethylhexylsalicylate.
GAF	Uvinul ms40	2.hydroxy.4.methoxy.5.sulphonic acid. benzophenone.
	Uvinul n539	2.ethylhexyl.2^1.cyano.3.3^1.diphenylacrylate.
	Uvinul 400	$2,4^1$.dihydroxybenzophenone.
Norda/Schimmel	Angstrol	Salicylates.
	Filtrosol A, B, Triple	Salicylates.
Naarden	Solisoline A, B	Unknown.
Merck	Eusolex 3573	4.phenyl.2.carbonicacid isoctyl ester.benzophenone.
	Eusolex 4360	Unknown.
	Eusolex 161	3.4.dimethoxyphenyl-glyoxylate Na.
	Eusolex 232	2.phenylbenzimidazoline.
Rhone-Poulenc	Rhoditan L	Salicylates.
Dragoco	Prosolal S9	Mixture of phenylacrylic and oxybenzoic esters
Merck	Eusolex 6653	Dibenzalazin.

TABLE 3-XI. CHEMICAL FORMULAE OF SEVERAL IMPORTANT SUNSCREENS.

Uvinul M-40 (GAF) **Eusolex 4360 (Merck)**  **2.Hydroxy-4.methoxy benzophenone**	**Uvinul D-50 (GAF)**  **2.2'.4.4'.Tetrahydroxy benzophenone**	**Uvinul DS-49 (GAF)**  **Sodium 2.2'.dihydroxy.4.4' dimethoxy.5.sulpho benzophenone**
Sunarome WMO (Felton) **Use: 5%**  **2.Ethylhexyl Salicylate**	**Escalol 506 (Van Dijk)** **Use: 2.5%**  **Amyldimethyl PABA**	**Escalol 106 (Van Dijk)** **Use: 2-3%**  **Monoglyceryl PABA**
Amerscreen P (Amerchol)  **(1 Mole + 3 Mole)** **Mixture of mono- and di-propoxylate of ethylPABA**	**Eusolex 232 (Merck)** **Novantisolsäure** **1-4%**  **2.Phenyl.benzimidazole-5.sulphonic acid**	**Eusolex 6300 (Merck)**  **3-(4.Methylbenzyliden) camphor**
GivTan F (Givaudin) **Cinoxate**  **2.Ethoxyethyl p.methoxy cinnamate**	**Parsol MCX (Givaudin)** **Neo Heliopan AV(Haarmann)**  **2.Ethylhexyl p.methoxy cinnamate**	**Guanin**  **2.Amino-6.hydroxy purine**

ations (24 brands) were collected. The identification was made mainly by TLC-GLC matching with industrial sunscreens as references and combined with chemical group reactions of two important classes of sunscreens. With the helpful cooperation of the chemical industry, some 27 sunscreens were collected for reference analysis. These reference sunscreens are given in Table 3-X, and the chemical formulae of several important sunscreens are given in Table 3-XI. This number of reference compounds, however, is far below the possibilities mentioned in literature and compiled in Table 3-IX. (Kuebler, 1968; Strobel & Inserra, 1968).

3.4.1. Thin-layer chromatography of sunscreens.

In the TLC system used, several dark spots from a single extract usually appeared under UV light. The following procedure is used to detect a sunscreen spot. The spot is scraped off, extracted with 3 ml of methanol, filtered and the UV absorption spectrum determined between 250 and 320 nm. At such a low concentration level (approximately 30 µg per 3 ml methanol, or 10 ppm) a sunscreen compound still has an absorption maximum near 300 nm. Other dark spots on the TLC-plate, such as from preservatives, will not have an appreciable absorption at 300 nm. See for examples Figure 3-H.

Extraction of the sample. Dissolve 1 g of the sample in 4 ml methanol. Heat gently. Add ca. 1 g anh.sodium sulphate. Mix thoroughly. Use upper methanol layer for TLC or GLC. Use 1% solutions of reference sunscreens in methanol. If the substance is not soluble in methanol, aid solution by adding small amounts of water until a clear solution is obtained.

Use self-made or precoated silicaplates with fluorescent indicator. If necessary activate plates at 105°C for ½ h. Use solvent system di-isopropylether - n.hexane - acetic acid (20 - 80 - 1, by volume). Develop in unsaturated tank. Visualize under UV light (254 nm).

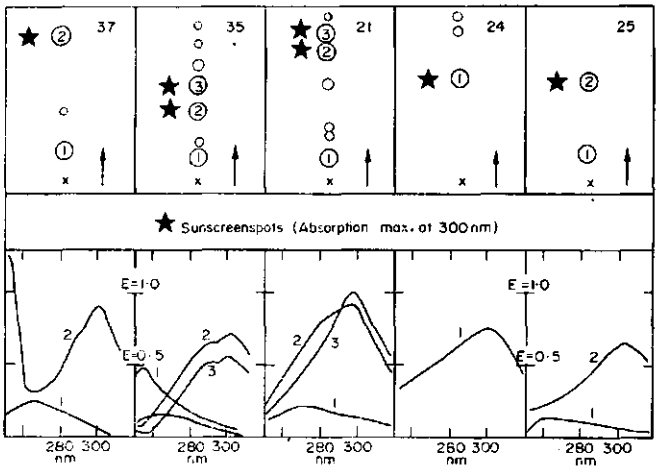
3.4.2. Gaschromatography of sunscreens.

GLC conditions. The same column for the analysis of aromatic amines (section 3.3.3) is used in the GLC of sunscreens. The column temperature is 230°C. Injection port 240°C. FID detector. Carrier gas: nitrogen.

Extraction of the sample is described under the TLC section (3.4.1). Inject ½-1 µl. Not all the reference sunscreens gave a response, but that was unimportant, as GLC matching was the main purpose of the test.

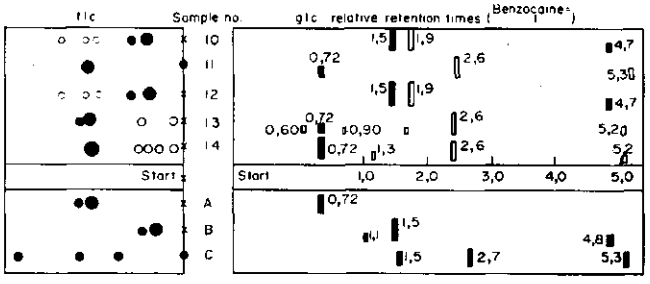
Alternative GLC systems for sunscreen compounds have been described by Davis (1966) and Paulus (1972).

FIGURE 3-M DETECTION OF A SUNSCREEN-SPOT ON A TLC-PLATE.



Detection of a sunscreen spot on a tlc plate. Scrape off the spot from the glass plate. Extract with 3 ml of methanol. Filter. Determine the uv spectrum of the solution in a 1 ml quartz cell, between 250 and 320 nm. If at this low concentration level (approx. 10 ppm) an appreciable absorption occurs at 300 nm, the tlc spot is a sunscreen. Examples: tlc of the samples no. 37, 35, 21, 24, 25; uv spectra of the spots.

FIGURE 3-1 EXAMPLE OF A TLC/GLC MATCHING FOR THE IDENTIFICATION OF A SUNSCREEN.



Example of tlc/glc matching for the identification of sunscreens. Sample no. 10 contains B (Givtan F). Sample no. 11 contains A (Eusolex 3573) and possibly C (Solprotex 1). Sample no. 12 contains B (Givtan F). Sample no. 13 and 14 contain A (Eusolex 3573) and possibly C (Solprotex 1).

3.4.3. Group reactions of several types of sunscreens.

Group reaction for p.aminobenzoic acid and esters. Ehrlich reagent, which contain 1% dimethylaminobenzaldehyde in 10% HCl, gives a strong yellow-orange colour. Apply directly on sample or as a visualization spray on a TLC plate.
NB. Many other compounds which are non-UV-absorbers, but with aromatic amine groups, show a positive Ehrlich reaction, e.g. sulphonamides.
To this group of sunscreens belong: p.aminobenzoic acid, ethyl p.aminobenzoate (=benzocaine), monoglyceryl p.aminobenzoate. The substituted esters, e.g. N.N.dimethyl p.aminobenzoic esters, do not give a reaction.

Group reaction for salicylic esters. The free salicylic acid is obtained after saponification of the esters. Boil $\frac{1}{2}$ g sample with 5 ml 8% ethanolic KOH under reflux for 30 min. Cool and dilute with 40 ml water. Neutralize to a pH 5-7. Add 1 drop 1% $FeCl_3$. A violet colour appears, which will persist after the addition of an equal volume of ethanol (phenol gives a violet colour that fades with ethanol).

3.4.4. Identification of sunscreens by TLC-GLC matching.

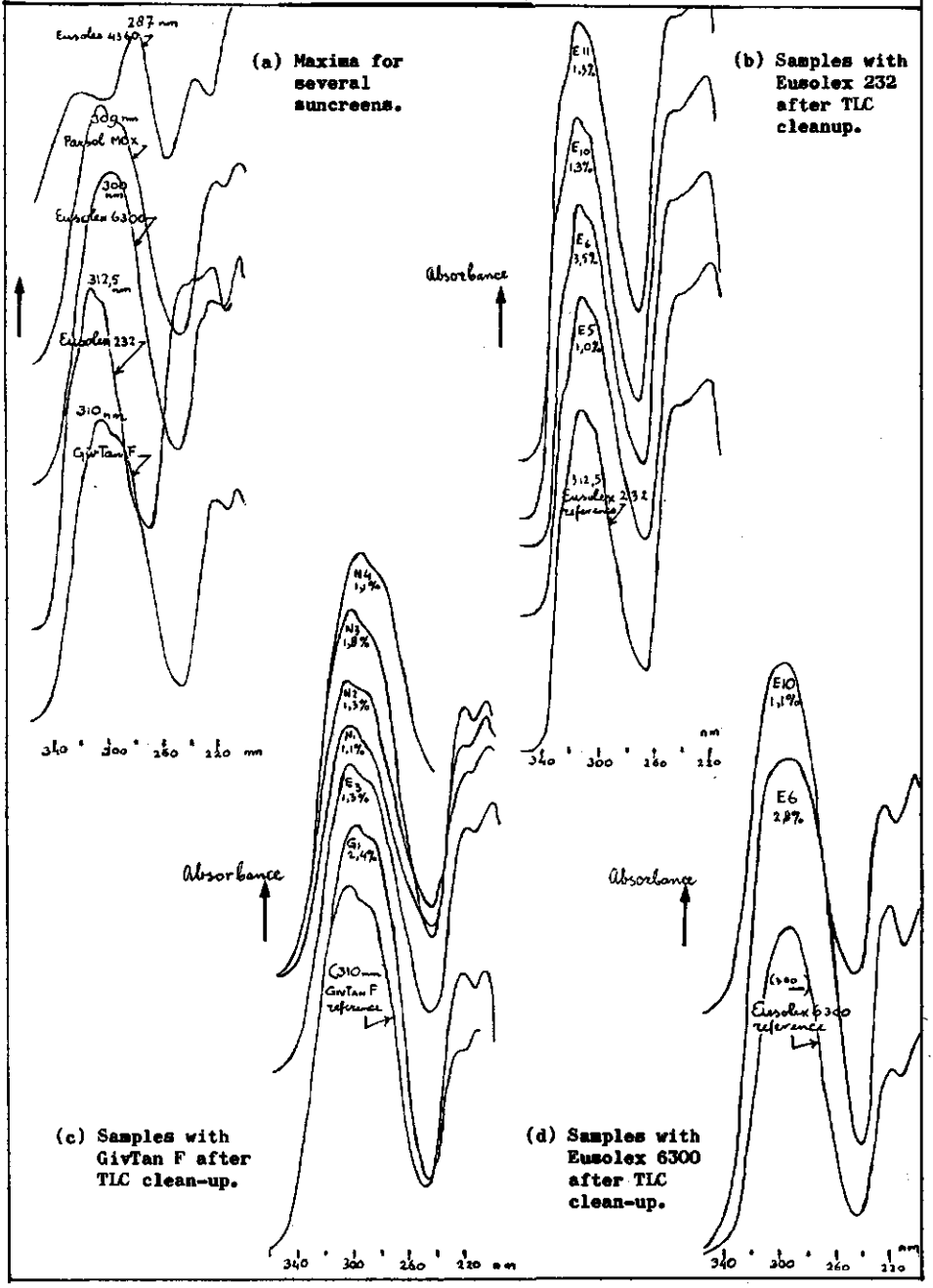
By means of simple TLC-GLC matching many of the sunscreens could be identified. An example is given in Figure 3-1. Not all the sunscreens could be identified, mainly because the range of reference compounds was not sufficient.

3.4.5. Quantitative determinations of sunscreens.

After identification of the sunscreens quantitative determination can easily be done by UV spectroscopy after TLC clean-up of the crude extracts. Reference standard sunscreens should be obtainable, particularly for the multi-component sunscreens. In such a case the sunscreen has to be chromatographed on the same plate and comparative measurement of the main component of the sunscreen have to be made. The following examples will be given for products using the sunscreens Giv-Tan F, Eusolex 232 and Eusolex 6300.

Experimental procedure. Shake 1 g product with some NaCl crystals and 5 ml of methanol. Filter and use filtrate for TLC. Use de-activated silica sheets with fluorescent indicator as described under section 3.7.2.2. A suitable solvent mixture should be used for the TLC clean-up procedure (examples: for Giv-Tan F and Eusolex 6300 use di-isopropylether-n.hexane-acetic acid (75 - 35- 1, by vol.) or ethylacetate - methanol - ammonia 35% (65 - 30 - 5, by vol.) for Eusolex 232). Apply 100 µl of the extract at the starting line over a length of approximately 4 cm. Apply the reference standard sunscreen onto the same plate, 100 µl of a 0.25% solution containing 250 µg sunscreen. Locate sunscreens under UV after

FIGURE 3-J UV-ABSORBANCE CURVES OF SEVERAL SUNSCREENS AFTER TLC PUTIFICATION.



development and mark the strongest band with a pencil of both the sample and the reference standard. Scrape off the silica from both bands and elute the UV-absorber compound as follows. For Giv-Tan F and Eusolex 6300 use methanol (10 ml) as extracting solvent. Shake and filter. Dilute filtrate further 5 times with methanol. Determine UV spectrum between 200-360 nm and measure absorbance at 310 nm for Giv-Tan F or at 300 nm for Eusolex 6300. For Eusolex 232 another solvent must be used, namely a mixture of 5 ml water + 5 ml methanol + 2 drops NaOH 4M. Dilute filtrate 5 times with methanol. Determine UV spectrum between 200-360 nm in a 1 cm quartz cell and measure absorbance at 312.5 nm. By comparing absorbances of sample extracts and reference standards the % of the sunscreens can easily be calculated. Examples are given in Figure 3-J. In principle any other sunscreen can be determined by this method, provided that reference compounds are obtainable. The curves (a) of Figure 3-J give the absorbance curves of the main band after TLC of five sunscreens.

3.4.6. Benzocaine in suntan preparations.

In several samples of two brands, the analytical data indicate the presence of benzocaine. This substance has sunscreen properties, but also acts as a local anesthesic. The presence of benzocaine was confirmed by its IR spectrum and its GLC response, after isolation by column chromatography.

Experimental details. Two samples (no 1 and 48) gave a strong orange colour with Ehrlich reagent. The samples were extracted as described under 3.4.1 and analysed by TLC, using the solvent mixture di-isopropylether - n.hexane - acetic acid (75 - 35 - 1, by vol.) and visualized by Ehrlich reagent. The following results were obtained:

		No 1	No 48
Benzocaine	Rf 0.55	+	+
p.Aminobenzoic acid	Rf 0.45	-	+
Monoglyceryl p.aminobenzoate	Rf 0	-	+

For confirmation the samples were saponified as follows. Boil ½ g of the sample with 5 ml 8% ethanolic KOH for 30 min. Cool and dilute with 20 ml water. Acidify with acetic acid to a pH of 1 - 3. Extract the free acid with 2 X 10 ml chloroform. Evaporate the chloroform fraction to 1 ml and use this for TLC. Only one spot, namely of the p.aminobenzoic acid should be present. Confirmation was also made by the IR spectra of the isolated benzocaine and by the GLC response of the same fraction. Isolation was done by column chromatography, using 2 x 20 cm glass columns filled with 15 g silicagel (diam.0.05 - 0.20 mm) suspended in n.hexane. Bring a mixture of 1 g sample + 1 g anh.sodium sulphate + 5 ml n.hexane on top of the column. Perform elution as given in Figure 3-L, which also gives information of the composition of the chromatographic fractions. The obtained IR spectra

and the GLC response of the benzocaine containing fractions are given on Figure 3-K.

3.4.7. Dihydroxyacetone in suntan preparations.

The browning agent dihydroxyacetone can be detected on TLC plates of cellulose by means of the same method as is used for the separation of sugars (Raadsveld & Klomp, 1971). It is interesting to note that with one of the samples this TLC system showed the presence of a mixture of synthetic organic colours (grey,red and yellow), which seemed to be used for artificial colouring of the skin.

Extraction of the sample. Dissolve 1 g of the sample in a mixture of 0.5 ml water and 3.5 ml methanol. Mix thoroughly. Use aqueous methanolic layer for TLC.

Reference solution of dihydroxyacetone is prepared by dissolving 400 mg of the substance in a mixture of 2 ml water and 23 ml methanol (1 µl = 20 µg dihydroxyacetone).

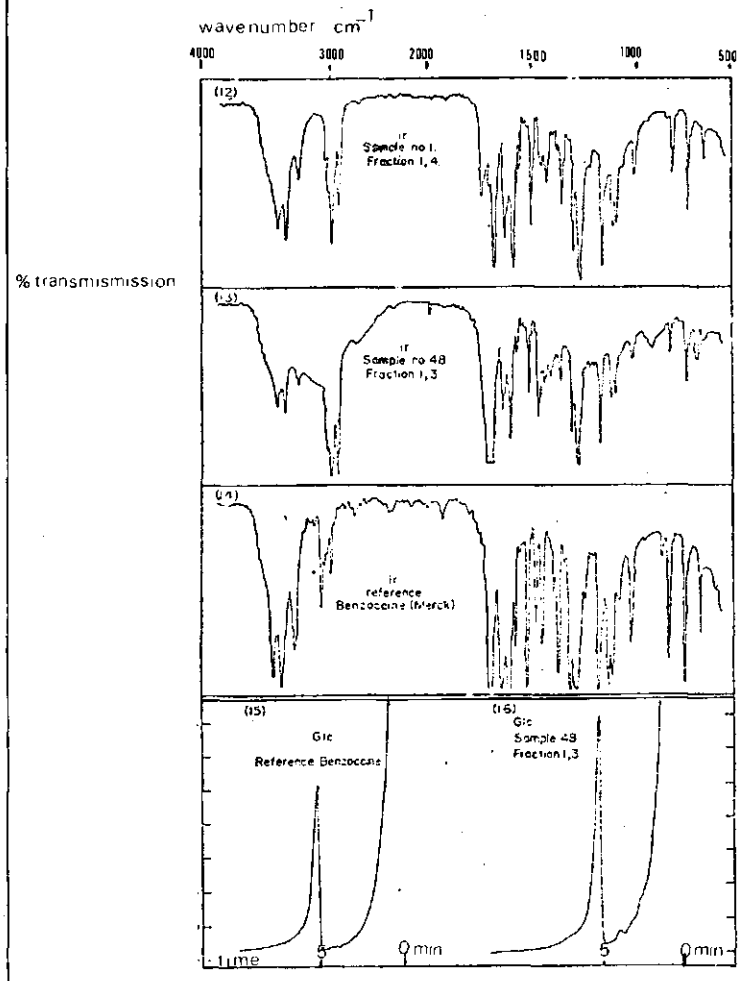
TLC plates were prepared as follows. For coating 5 plates of 20 X 20 cm mix 15 g cellulose MN 300 with 90 ml of water. Dry at 90°C until thoroughly dry. The following solvent mixture is used for TLC: water - ethylacetate - pyridine (15 - 60 - 25, by vol.). Use unsaturated tank. Time about 45 minutes for a 15 cm path. Spot 10 µl of the extract and 5 µl of the reference solution for TLC. Visualize by spraying with a mixture of 1.3 ml phosphoric acid 85% + 0.93 ml aniline + 100 ml ethanol 70%. Heat the plates at 100°C for 15 minutes.

Dihydroxyacetone	Rf	0.85 . Colour tomato-red.
Glucose	Rf	0.30 . Colour brown.
Rhamnose	Rf	0.60 . Colour green.

3.4.8. Analytical results of the "suntan preparations" market survey.

The analytical results of a range of commercial products are given in Table 3-XII. In 20% of the samples more than one sunscreen is used which might be present in both phases of the emulsion. From the TLC data the conclusion can be drawn that many of the industrial sunscreens as offered by the chemical industry are not always single compounds, but mixtures of 2,3 or sometimes 5 components. The browning agent dihydroxyacetone was found in 8 samples of 4 brands. Benzocaine was found in 6 samples of 2 brands, but lidocaine has not been detected. Due to an incomplete library of reference compounds several sunscreens remain unidentified.

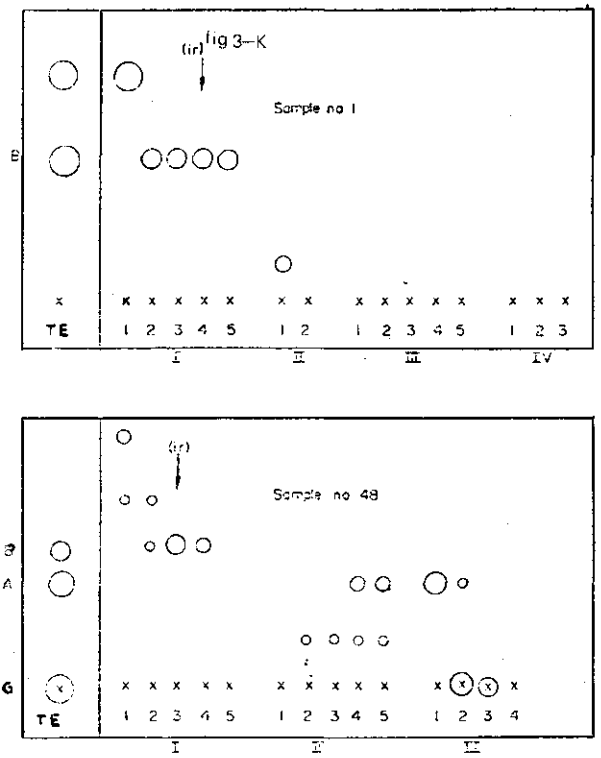
FIGURE 3-K CONFIRMATION OF BENZOCAINE BY IR-SPECTRA AND GLC.



Fraction numbers refer to Figure 3-L.

GLC conditions are according to section 3.4.2.

FIGURE 3-L TLC-PATTERNS OF COLUMN-CHROMATOGRAPHIC FRACTIONS OF SAMPLES CONTAINING BENZOCAINE.



Sample 1: I = 1 x 60 ml n.hexane and 4 x 20 ml n.hexane-ether (1-1 vol.)
II = 2 x 20 ml ether-methanol (3-1,vol.)
III = 5 x 20 ml ether-methanol (1-1,vol.)
IV = 3 x 20 ml methanol

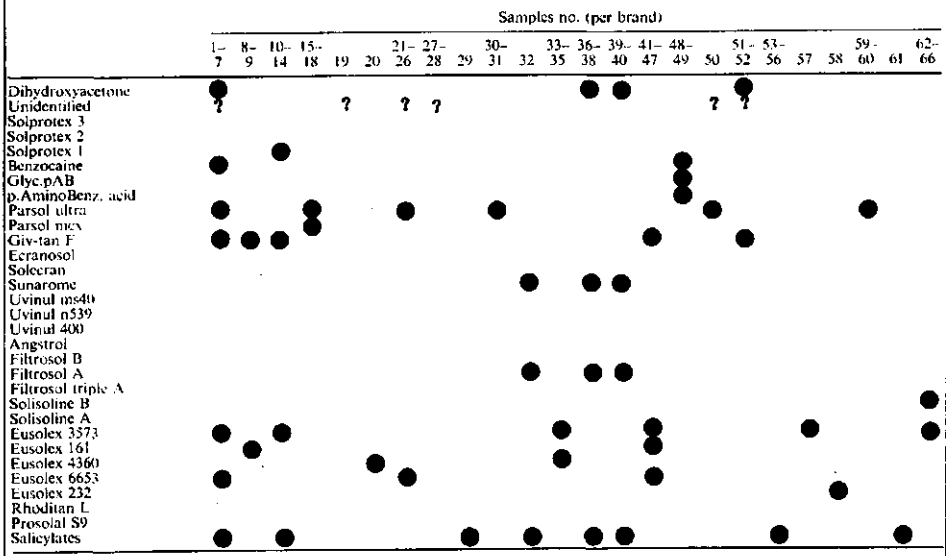
Sample 48: I = 1 x 60 ml n.hexane, and 4 x 20 ml n.hexane-ether (1-1,vol.)
II = 5 x 20 ml n.hexane-ether (1-2,vol.)+ ½ ml acetic acid.
III = 4 x 20 ml methanol

TE = total extract. A = *p.aminobenzoic* acid

B = benzocaine G = monoglyceryl p.aminobenzoate

TABLE 3-XII

ANALYTICAL RESULTS OF THE SUNTAN PRODUCTS MARKET SURVEY. 1971



3.5. Propellants and solvents in aerosol cosmetics.

Aerosol cosmetics which have an important place in our society, contain major proportions of propellants and solvents. These compounds have toxicity hazards if inhaled and could also be fire hazards because of the flammability of several of the compounds. It was therefore of interest to study the aerosol cosmetics on the market in order to estimate the real danger of these products. The composition of some 60 samples of hairspray aerosols was examined in spring 1973. GLC analysis is the method of choice and some excellent papers have been published in this field (Jenkins & Amburgey, 1959 ; Bourne & Murphy, 1969). Several columns have been described which give a satisfactory resolution of the propellants and solvents used. The difficulty of the analysis, however, is in the sample handling prior to the GLC analysis.

To transfer the pressurized liquid of an aerosol from the container to the injection port of the gas-chromatograph, one can use a closed system as described by Cannizzaro & Lewis (1969). The aerosol can is pierced below the liquid level by a special "can-piercing unit". The pressurized liquid is then led , via a metering liquid sampling valve, to the injection port of the gas-chromatograph. An alternative way of bringing the pressurized liquid to the gas-chromatograph is by using an intermediate transfer vessel made of glass, without damaging the can, and injecting the pressurized liquid by means of a special syringe. Schubert & Keitel (1972) used a small glass serum bottle for this intermediate transfer. In this study the following procedure was used:

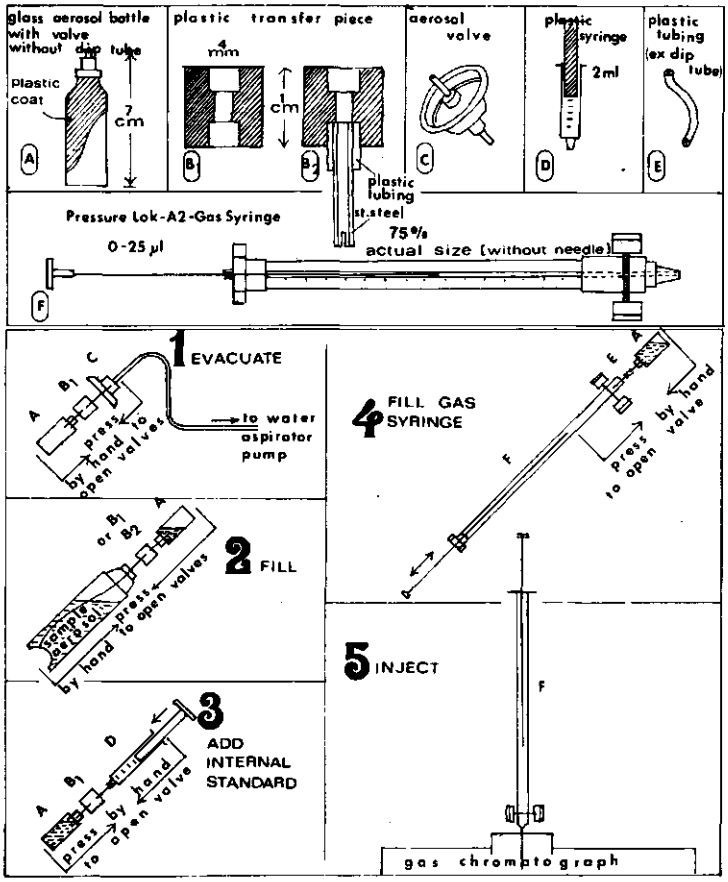
3.5.1. Sampling.

Figure 3-M is a schematic diagram of the aerosol sampling procedure.(A) is a small glass aerosol bottle - 20 mm x 50 mm (no 542 Henri Desjonqueres, 81 rue Talbout, Paris 9, France); (B) shows plastic transfer pieces of polyethylene buttons (DPV, Hattersheim, W.Germany); (B1) is used for containers with a male valve and (B2) for containers with a female valve, and the same as B1 with extra polyethylene and stainless steel tubing; (C) is an aerosol valve (plate 31-6402, cone 04-1210, casing 07-7710, DVP); (D)is a disposable 2ml polyethylene syringe without needle and for hospital use; (E)is a polyethylene aerosol dip tube; (F) is a gas-syringe (0 to 25 µl), with sliding teflon valve at end (Pressure-Lok no A2, Precision sampling, Baton Rouge, La, USA).

Evacuate, weigh and cool the small glass aerosol bottle (A). Evacuation by a water aspirator pump is sufficient. Use tranfer pieces(B1)and (C). Remove spray nozzle of aerosol sample. Transfer ca. 8 ml aerosol liquid from original container to (A), using items(B1) or (B2)(depending on valve system of original container)

FIGURE 3-M

SCHEMATIC DIAGRAM OF AEROSOL SAMPLING PRIOR TO GAS-CHROMATOGRAPHY.



and (E). Weigh accurately and calculate weight of transferred sample (= m gram)

Introduce ca. 1 ml internal standard (cyclohexane) into (A), using syringe (D) (without needle) and transfer piece (B1). Weigh accurately and calculate weight of cyclohexane (= s gram). Phase separation might occur if too much cyclohexane is added. In that case repeat sampling from beginning and reduce amount of cyclohexane to obtain clear single phase mixtures.

Fill special gas syringe (F) as follows: Invert small glass aerosol bottle; connect it to gas syringe without needle and push syringe valve to "open" position with thumb. Slowly move piston up and down several times, taking care that the piston reaches the syringe bottom completely each time. This is an extremely important operation and should be carefully done in order to take a representative sample of mixture. No "gas space" should be present in the syringe. Pre-flushing syringe on outside with propellant 12 (dichlorodifluoromethane) will aid the operation. If syringe space is completely filled with representative sample of the mixture, close syringe valve with thumb at ca. 20 µl. Check periodically for leaks in syringe system at this stage. Wait several minutes while holding syringe piston in a fixed position. If some gas space appears in syringe, then either piston or valve is leaky and the syringe should be repaired. Attach needle to syringe and inject into septum of injection port of the gas-chromatograph. Immediately and succesively push syringe valve to position "open" and push down piston to 0 level. Proceed with gas-chromatographic analysis.

3.5.2. Gas-chromatography of propellants and solvents.

The GLC system used is the same as that used by Cannizzaro & Lewis (1969), with small modifications. The column is only 4 m in length. A pre-column is used to prevent fouling of the main column. The resolution of the solvents and propellants of hairspray aerosols is satisfactory, except for the propellants 12/114 and for isobutane/dimethylether.

GLC conditions. Gas-chromatograph with catharometer detector (bridge current 150 mA) and temperature of 200°C. Temperature of the injection port: 125°C. Pre-column attached before main column: 30 cm in length, 4 mm intern diam, stainless steel and filled with Chromosorb WHP 60/80. To be replaced after ca. 100 injections. Main column: 400 cm X 4 mm, stainless steel, filled with 20% Hallcomid M18 on Chromosorb WHP 60/80. Column temp. 65°C isotherm. Carrier gas: helium 50 ml/min. Time per injection approximately 30 min. Examples for retention times (in min.):

Propane	1.9	Propellant 11	5.1	Cyclohexane	18.4
Propellant 12	2.0	n.Butane	2.8	Ethanol	20.4
isoButane	2.2	Acetone	8.1	Methylchloroform	23.0
Dimethylether	2.2	Methanol	12.5	isoPropanol	25.5
		Dichloromethane	14.6		

3.5.2.1. Reliability of the proposed injection system.

How reliable is a GLC-injection with the system ? To answer this question one transfer vessel was filled with a hairspray aerosol sample (+ internal standard) and 10 succesive injections of 20 µl were made. From the integrated GLC-data the % of solvents and propellants were calculated. Statistical evaluation gave the following variation coëfficients: P12 (1.4%), P11 (1.3%), DCM (1.5%), EtOH (2.5%). See Table 3-XIII. It can be concluded that the sampling and injection system is quite reliable.

3.5.2.2. Composition change of an aerosol during use.

Can an aerosol sample be analysed at any filling stage or, in other words, will the composition of an aerosol change during use ? To find the practical answer one container was sampled several times according to the method, while releasing it between sampling. The results of the analysis of the five aerosol samples in the intermediate vessels are given in Table 3-XIV, which proves that sampling can be done at any filling stage, except when the can is nearly empty.

3.5.2.3. Importance of the injected volume.

Is the injected volume of importance ? Generally speaking, the introduction of an internal standard makes the volume of the injected liquid of no importance. But in this kind of analysis another important factor must be considered, namely that the dead volume of the needle must be negligible in comparison to the injected volume. Experimentally the minimum volume was (for our GLC conditions) 15 µl. In all our experiments the volume was standardized to 20 µl to obtain optimal reliability.

3.5.2.4. Cyclohexane as a general internal standard.

Is one general standard (cyclohexane) acceptable for such a mixture of different kinds of compounds (alkanols, alkanones, hydrocarbons, fluorinated and chlorinated hydrocarbons) ? The linearity of the response for certain practical concentration levels was checked by analysing three different reference mixtures. The calculated results are given in Table 3-XV from which it can be concluded that for these levels of concentration cyclohexane is acceptable as a general internal standard in our procedure.

TABLE 3-XIII RELIABILITY OF SUCCESIVE INJECTIONS OF THE SAME MIXTURE.

One small (10 ml) all-glass aerosol bottle was filled with a hairspray aerosol sample and an internal standard added. The liquid mixture was analysed by 10 successive injections according to the method and the integrated peak areas were used to calculate the "% of the volatile components. The calculated "% are:

1	27.4% P12	54.8% P11	5.25% Dichloromethane	6.76% Ethanol
2	27.1	54.5	5.16	6.79
3	27.3	55.0	5.22	6.91
4	27.5	55.2	5.45	7.21
5	27.2	55.5	5.25	7.14
6	26.8	54.1	6.34+	6.74
7	26.5	53.5	5.76+	6.69
8	27.3	55.1	5.23	7.10
9	27.8	55.9	5.27	7.02
10	27.3	55.0	5.24	6.92
Mean	27.2	54.9	5.26	2.69
Standard deviation	0.4	0.7	0.08	0.20
Variation coefficient	1.4%	1.3%	1.5%	2.5%

+ Abnormal results.

TABLE 3-XIV COMPOSITION OF AN AEROSOL HAIRSPRAY DURING USE.

A single hairspray aerosol is sampled five times for analysis, at different stages of emptying. The following data were obtained:

	'Full' ≈ 530 g net weight	Removed 200 g	Removed 300 g	Removed 400 g	'Empty' 30 g left
Propellant 12	29.8%	29.4%	29.3%	28.5%	23.8%
Propellant 11	45.0	45.1	45.1	45.8	46.6
Acetone	0.30	0.29	0.30	0.23	0.28
Dichloromethane	22.7	22.6	22.7	22.5	26.6
Ethanol	4.4	4.4	4.0	4.1	4.7

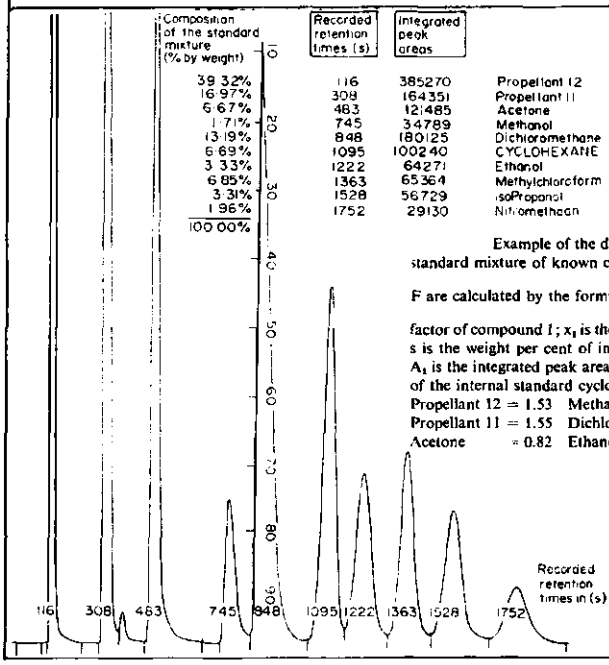
TABLE 3-XV RELIABILITY OF CYCLOHEXANE AS A GENERAL INTERNAL STANDARD.

Three different standard mixtures, containing nine compounds and cyclohexane were analysed by glc. From the integrated peak areas the correction factors F were calculated by the formula $F = x_i/S)\,(A_s/A_i)$.

	% by weight			Correction factors			Concentration levels
	A	B	C	A	B	C	
Propellant 12	39.3	21.4	28.4	1.52	1.53	1.47	20–40%
Propellant 11	17.0	8.9	26.6	1.58	1.55	1.53	10–30%
Acetone	6.7	18.0	13.3	0.86	0·84	0·80	5–20%
Methanol	1.7	4.6	3.2	0.77	0.74	0.72	1–5%
Dichloromethane	13.2	18.0	6.6	1.19	1.12	1.10	5–20%
Cyclohexane	6.7	6.1	6.6	1	1	1	
Ethanol	3.3	4.5	1.9	0.78	0.81	0.79	2–5%
Methylchloroform	6.8	12.4	9.9	1.55	1.57	1.57	5–15%
isoPropanol	3.3	4.6	1.9	0.84	0.87	0.84	1–5%
Nitromethane	2.0	1.5	1.6	1.03	1.00	1.18	1–2%

Conclusion: For these levels of concentration cyclohexane as a general internal standard is acceptable, except for nitromethane.

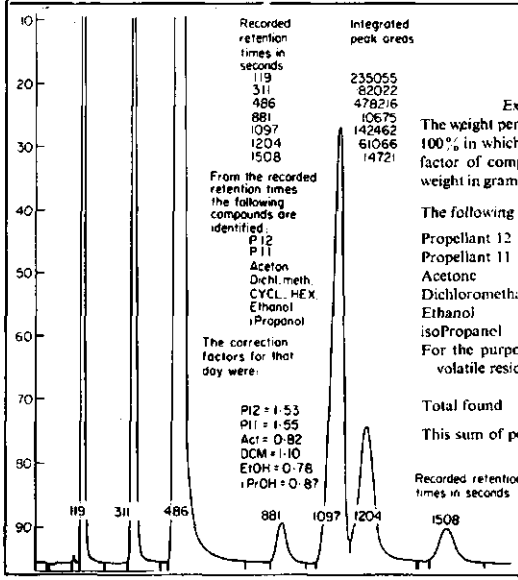
FIGURE 3-N **AN EXAMPLE OF THE DETERMINATION OF CORRECTION FACTORS**



Example of the determination of correction factors F. 20 µl of a standard mixture of known composition are injected. The correction factors F are calculated by the formula: $F_i = \frac{x_i}{s} \cdot \frac{A_s}{A_i}$, in which F_i is the correction factor of compound i; x_i is the weight per cent of compound i in the mixture; s is the weight per cent of internal standard in the mixture (Cyclohexane); A_i is the integrated peak area of compound i; A_s is the integrated peak area of the internal standard cyclohexane. The calculated correction factors are:

Propellant 12	= 1.53	Methanol	= 0.74	Methylchloroform	= 1.57
Propellant 11	= 1.55	Dichloromethane	= 1.10	isoPropanol	= 0.87
Acetone	= 0.82	Ethanol	= 0.78	Nitromethane	= 1.01

FIGURE 3-O **EXAMPLE: GLC ANALYSIS OF AN AEROSOL HAIRSPRAY.**



Example of the glc analysis of a hairspray aerosol sample no. 34

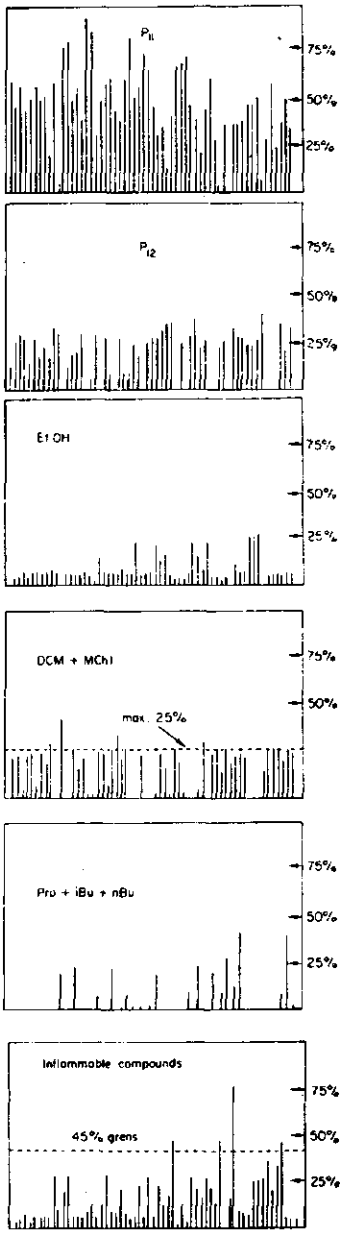
The weight per cent were calculated with the formula $\% = (A_i \cdot F_i / A_s) \cdot (s/m) \cdot 100\%$ in which A_i = integrated peak area of compound 1; F_i = correction factor of compound 1; A_s = integrated peak area of Cyclohexane; s = weight in grams of Cyclohexane; m = weight in grams of the sampled mixture.

The following data were obtained:

Propellant 12	35.5%
Propellant 11	12.7%
Acetone	39.3%
Dichloromethane	1.2%
Ethanol	4.9%
isoPropanel	1.3%
For the purpose of analytical control, we determined the non-volatile residue by gravimetric analysis and found—Residue	1.9%
Total found	96.8%

This sum of per cent differs less than 5% from the 100%, so the analysis is acceptable.

FIGURE 3-P ANALYTICAL RESULTS: MARKET SURVEY OF AEROSOL HAIRSPRAY (1973)



Propellant 11 (Trichlorofluoromethane) has a boiling point of 23·8°C. The histogram profile shows clearly that this compound is the most important propellant/solvent in hairspray aerosols. Average % between 30 and 60%; in 4 samples more than 80%!

Propellant 12 (Dichlorodifluoromethane) is an important propellant in hairspray aerosols. Found in 80% of the samples; average 20–25%.

Dimethylether: Two samples contain dimethylether which is only recently introduced as a propellant.

Ethanol is an important solvent. Almost all samples contain ethanol at a level of 10%. Some samples contain 25%, but higher levels do not occur.

Methanol. Small amounts (less than 0·5%) are found in 45% of the samples, probably as a denaturant of ethanol or isopropanol.

Dichloromethane + methylchloroform (DCM+MChl): These chlorinated solvents are used instead of *ethanol to lower the price of the hairspray aerosol.* The Dutch Cosmetic Bill states a maximum of 25%. Two samples contain more, namely 42·8% and 34·2%. A quarter of our samples do not contain chlorinated solvents.

Hydrocarbons (Propane+nButane+iButane) are used as propellants in 25% of the samples. In one sample even more than 40%, but the average % is 20%.

Inflammable compounds (Sum of hydrocarbons-dimethylether-aceton-methanol-ethanol-isopropanol). Only four samples exceed the 45% inflammable compound which is accepted by aerosol technologists as an upper limit for the fire safety of aerosols.

3.5.2.5. Practical examples.

The correction factors F were obtained by simple injection of standard mixtures of which the composition is known exactly. Several standard mixtures were made up and stored in 100 ml glass aerosol bottles without dip tubes, so that they could be used over and over again. The analysis of such a mixture and the calculation of the correction factors F is given in Figure 3-N. The correction factors should be determined daily for optimal reliability. They might vary a little, depending on the slightly different GLC conditions day by day.

Since every injection on the gas-chromatograph takes 30 minutes for total response, sampling in the intermediate transfer vessels can be done in the meantime, including the addition of the internal standard. 20 µl of each sample should be injected and the % calculated from the integrated data as shown in Figure 3-O. By this single injection a total quantitative analysis of the propellants and solvents is accomplished within half an hour.

A simple analytical control has been devised for each determination, namely by summation of the found %, including the % of the non-volatile residue which should be determined separately by gravimetric analysis. If the sum of the percentages differs more than 5% from the theoretical 100%, the analysis including the sampling procedure should be repeated. This simple control has proved invaluable during our analytical survey, as faulty analysis could then be avoided.

3.5.3. Analytical results of the "aerosol hairspray" market survey.

The results of the analysis of 60 hair-spray aerosol samples can be seen in Figure 3-P, which shows histograms of the levels found in the samples. Sampling from the market was done in 1973.

3.6. Hormonal substances.

Since the discovery of Zondek (1929) of percutaneous absorption of oestrogenic hormones on human skin and its beneficial effects on wrinkles and skin texture, the cosmetic industry is very much interested in the use of hormonal substances in its products. Several papers (Scherm, 1966; Everse, 1973 ; Masters, 1974) and patents (Masters, 1961) have appeared since then. Some cosmetic effects other than anti-wrinkle action have been claimed, such as the development of the female breast by

oestrogens and gestagens (McBryde, 1939), female hair growth depression by oestrogens and male hair growth promotion by androgens, including beard and moustache (Papa, 1972; Turner 1971).

The use of hormonal substances is not without risks. The hazards depend very much on the concentration levels of these pharmacologically very active compounds (Everse, 1973). It is therefore important to develop biological and chemical methods for detecting the hormonal activity, identifying the compounds and determining the levels of concentration.

Biological methods have a general character. The biological method for oestrogen activity for instance is valid for the female sex hormones and other natural steroidal oestrogens, as well as for the synthetic oestrogens (such as DES, hexoestrol) and the numerous phyto-oestrogens of the plant kingdom. Chemical methods on the contrary focus the analysis on a small, distinct number of oestrogenic compounds, carefully selected from the literature. Identification of the active compound is therefore possible in many cases. The chemical methods of this study were focused on 8 hormonal substances, which have been selected from the literature.

3.6.1. Sampling of market products.

About 50 samples were purchased from the Dutch market in the second half of 1973. Since no formula was stated on the labels or on the information pamphlets, selection of the cosmetic products was based on their beautifying claims or cosmetic action, such as: a. anti-wrinkle creams for the face and sometimes specifically for the neck and around the eyes, indicated for the older woman, with visible results after about two weeks of daily application.; b. promotion of growth of the female breast; c. depression of female hair growth, such as hirsutism; d. promotion of male hair growth (beard, moustache).

Prices of the samples varied from 3 to 40 US $ per unit.

3.6.2. Biological methods.

The biological method for determining oestrogenic activity of cosmetic samples (Liem & Huis in 't Veld, 1976) is based on the work of Allen & Doisy (1923). Concentration levels as low as 5 µg oestradiol-17β per g (5 ppm) can be detected by the proposed method, which uses the cosmetic product as such. Such levels are far below the so-called "cosmetic level" of 35 µg per g. Androgenic activity in cosmetics can be detected by biological methods at levels of 0.2% testosterone (Huis in 't Veld, 1975), but failed with products containing 0.02%. The method of determining gestagenic activity in cosmetic products is still being studied at the Nat-

ional Institute of Public Health, dept. of endocrinology.

3.6.3. Chemical methods.

The proposed chemical methods were focused on 8 hormonal compounds selected from technological information. (a) four of the most important oestrogenic compounds: oestradiol-17β or its esters, oestrone, oestriol and the synthetic non-steroidal compound diethylstilboestrol (DES) or its esters. All four compounds have a phenolic group. (b) two gestagenic compounds: progesterone and ethisteron (ethinyl testosterone). (c) pregnenolone or its acetate, which is a progesterone-precursor. (d) testosterone or its ester as the most important androgenic compound.

Many papers in clinical and biochemical fields deal with the analysis of these hormonal compounds. Lisboa and Dicsfalucy (1962) have described the separation and characterization of many steroidal oestrogens by TLC. Adlercreutz & Luukainen (1968) use gas-chromatographic techniques for determining oestrogens in biological fluids. The most sensitive technique is the radio-immunological assay method (deJong et al., 1974). The choice of methods for analysing cosmetics depends very much on the concentration levels of the active compounds. High levels (500-5000 ppm) can easily be determined by chromatographic methods after simple extraction. Low levels oestrogens (ca. 35 ppm), however, need a clean-up step before the actual chromatographic determination. Fortunately these oestrogens are phenolics, which can easily be purified by the acid-base solvent extraction procedures (Smith & McNeill, 1972). At pH 1 these phenolic oestrogens move to the phase when shaken with a water-immiscible solvent. At pH 10.5 the phenolic steroids remain in the organic phase, permitting the removal of a great quantity of interfering substances. At pH 13 the phenolic steroids moved to the aqueous phase. An alternative clean-up procedure is column-chromatography, as described by Jones et al. (1968). For identification TLC is the most convenient and generally applicable method. Visualization in case of samples with low-level oestrogen is however a problem, as too many interfering compounds of the cream matrix respond to the chromogenic sprays. Only for samples containing DES is a more specific way possible, namely by UV radiation (Schuller, 1967), which converts DES into a yellow phenanthrene derivative, which fluoresces under long-wave UV light (yellow-brown). For the steroidal oestrogens we find a GLC confirmation very useful. GLC alone is however not practicable. GLC-data of a natural mixture of silylated steroidal compounds clearly shows that GLC alone cannot make a good identification. Combinations of TLC and GLC generally result in reliable results.

3.6.3.1. Outline of the analysis.

The outline of the analysis given in Table 3-XVI is mainly based on the inform-

TABLE 3-XVI. ANALYTICAL GUIDE FOR HORMONAL SUBSTANCES IN COSMETICS.
(based on claimed action of the products)

Product action claim.	Possible active hormon. ingred.	Conc. level (%)	Chemical procedures (see text)	
			Qualitative	Quantitative
●Anti-wrinkle	DES or DES-esters	0.0035	A B H I	A B H I or C E J K or A B E J K
	Steroidal oestrogens:	0.0035	A B H or	A B D J K or
	a. Oestradiol-17b (E2)		A D H	C D J K
	b. E2-dibenzoate			
	c. Oestrone.			
	d. Oestriol.			
	Gestagens:			
	a. Progesterone	0.0100	A H	A H
	b. Ethisterone	0.0400	A H	A H
	(NB. Progesterone might be combined with "low-level" oestrogen)			
	Other steroidal cmpd.			
	Pregnenolone or its acetate.	0.2000	A H	F K
● Development of female breast.	DES or DES-esters	0.1000-0.2000	A H I	A B H I or A E J K or C E J K
	Steroidal oestrogens:			
	a. Oestradiol-17 b (E2)	0.1000-0.2000		
	b. E2-dibenzoate.			
	c. Oestrone.			
	d Oestriol.			
● Depression of abnormal hair growth in the female.	DES or DES-esters	0.0100-0.1000	A H I	A B H I or A E J K or C E J K
● Promotion of male hair growth	Testosterone or its propionate.	0.2000	A H	A H

ation of each product regarding its claimed cosmetic action. The following procedures can be used in connection with that analytical guide.

3.6.3.2. Extraction.

Three extraction procedures (A,B and C) are described.
(A) General extraction procedure. Weigh 2 g of the sample. Add 10 ml toluene. Distil off the azeotrope toluene-water in a rotational evaporator under reduced pressure. Add 10 ml ethanol 96%, 10 ml acetonitrile and 20 ml pentane to the hydrated mixture. Shake vigorously and wait until a good separation of the phases has occurred. Discard the pentane fraction. Evaporate the acetonitrile-ethanol fraction and dissolve the residuein 4 ml of chloroform. (Strength of the extract : 1 ml = 0.5 g sample).
(B) Extraction for low-level, free phenolic oestrogens. Use 2 ml of extract A. Evaporate solvent. Dissolve residue in 5 ml NaOH 4M. Shake succesively with 2 x 5 ml chlorofrom and 1 x 5 ml pentane. Discard organic layers, if necessary after centrifuging. Acidify aqueous layer with HCl 4M to pH 1. Shake with 10 ml chloroform. Discard aqueous fraction. Dry with anh. sodium sulphate. Evaporate chloroform. Dissolve residue in 0.25 ml chloroform. (Strength of the extract: 1 ml = 4 g sample).
(C) Extraction for low-level, esterified phenolic oestrogens. Mix 1 g of the sample with 20 ml ethanolic KOH 0.5M. Boil mixture under reflux for half an hour. Cool, dilute mixture with 30 ml of water. Evaporate ethanol on a steambath. Adjust the volume to approximately 25 ml. Add 10 g of sodium chloride. Shake vigorously to dissolve as much as possible of the salt. Cool to 10°C and add 0.5 g hyflosupercel (filtering aid). Filter. Wash residue on filter with 2 x 10 ml NaOH 0.5M, which has previously been cooled to 10°C. Shake the clear combined filtrates with 2 x 50 ml chloroform. Wait until the separation of the phases has occurred, if necessary centrifuge. Discard the chloroform layers. Acidify aqueous fractions with HCl 4M to pH 1. Shake the phenolic oestrogens with 2 x 25 ml chloroform. Dry with anh. sodium sulphate. Reduce volume to 0.25 ml by evaporation. (Strength 1 ml strength = 4 g sample).

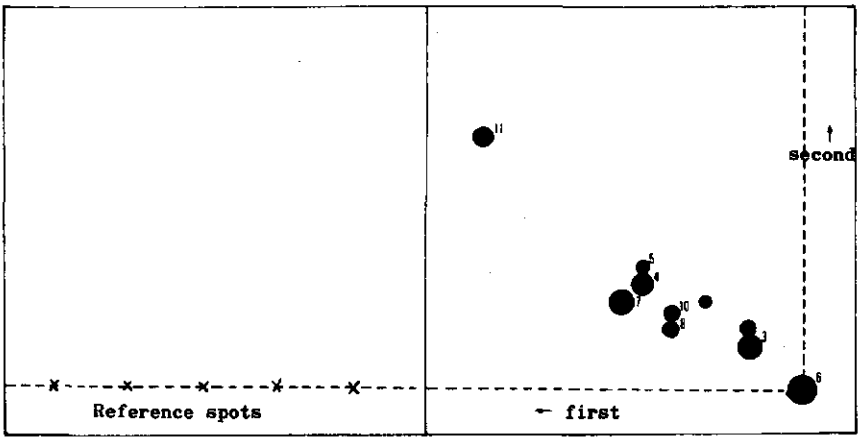
3.6.3.3. Clean-up.

Three alternative column clean-up procedures are given here.
(D) General column clean-up. Mix 2 ml of extract A (= 1 g sample), 5 g anh.sodium sulphate and 3 g hyflosupercel to a homogeneous mass. Put this mixture on top of column (diameter 18 mm, glass), filled with 5 g alumina (neutral, activity II according to Brockmann). Elute succesively with: fraction I a mixture of 85 ml n.hexane + 15 ml chloroform. Discard this fraction. Fraction II a mixture of 50 ml n.hexane + 50 ml chloroform. This fraction will contain oestradiol-17β benzoate ,

TABLE 3-XVII. TLC OF HORMONAL SUBSTANCES : Rf AND VISUALIZATION TABLE. AND TWO-DIMENSIONAL PATTERN.

compound	solvents*			colours*	
	1	2	3	initial	final
1. Trans-DES	0.38	0.50	0.52	purple	purple
2. Cis-DES	0.35	0.45	0.35	purple	purple
3. Oestradiol	0.18	0.28	0.20	light green	ultra-marineblue
4. Oestradiol benzoate	0.29	0.32	0.42	light green	ultra-marineblue
5. Oestrone	0.30	0.35	0.40	blue	Berlinblue
6. Oestriol	0	0.02	0.04	grey	grey
7. Progesterone	0.28	0.40	0.49	orangebrown	purple
8. Ethisterone	0.20	0.20	0.33	redbrown(hot) reversible colour change	olivegreen (cold)
9. Testosterone propionate	0.50	0.60	0.72	brown	brown
10. Pregnenolone	0.24	0.33	0.33	green	bluegrey
11. Pregnenolone acetate	0.65	0.80	0.88	green	bluegrey

*See section 3.6.3.4. under G.



testosterone propionate, progesterone, pregnenolone acetate. Fraction III 100 ml ethanol 96%. This fraction will contain ethisterone, oestradiol-17β, oestrone and oestriol.

(E) Column clean-up for DES. Use 0.25 ml of extract B (= 1 g sample). Put this on top of column (6 cm x 7 mm), filled with 3.5 g alumina (basic, activity IV according to Brockmann). Elute succesively with: fraction I 10 ml chloroform. Discard the fraction. Fraction II 5 ml benzene. Discard the fraction. Fraction III a mixture of 13.5 ml acetone + 1.5 ml water. This fraction will contain DES.

(F) Column cleanup for pregnenolone acetate. Mix 1 g sample with 3 g anh.sodium sulphate and 2 g hyflosupercel and 2 ml n.hexane. Put this on top of column (diameter 18 mm, glass) filled with 15 g alumina (neutral, activity II according to Brockmann). Elute succesively with : fraction I a mixture of 85 ml n.hexane + 15 ml chloroform. Discard this fraction. Fraction II a mixture of 50 ml n.hexane+ 50 ml chloroform. This fraction will contain pregnenolone acetate. Evaporate fraction II to 1 ml.

3.6.3.4. Thinlayer-chromatography.

Three procedures will be described here.

(G) 1-dimensional TLC . Use commercial silica TLC sheets with fluorescent indicator. Solvent 1: n.hexane-ethylacetate (70-30,by vol.). Unlined tank. Solvent 2: n.hexane-benzene-ethylacetate (70-20-20, by vol.). Unlined tank. Solvent 3:n.hexane-ethylacetate (60-40, by vol.). Unlined tank. Optimal amounts of the hormonal substances: 1 µg. Time approximately 30 minutes for a 15 cm path. Visualization: mixture of anisaldehyde and sulphuric acid conc. and acetic acid (1-2-97 by vol.). Spray until wet. Heat at 130°C for 3-5 minutes, until optimal colours have appeared. Approximate Rf values and characteristic colours are given in Table 3-XVII.

(H) 2-dimensional TLC. Use commercial silica TLC sheets without fluorescent indicator 10 x 20 cm, lined as described in Table 3-XVII. Solvent for first direction n.hexane-ethylacetate (60-40, by vol.). Solvent for the second direction n.hexane-ethylacetate (70-30, by vol.). Left section of plate is for reference spots.

(I) Visualization of DES. This method is very specific for DES. Expose spot to short-wave UV light (for instance Philips TUV, 15 watt) for 3 minutes. DES will be transformed into yellow phenanthrene derivative, which gives a yellow-brown fluorescence under long-wave UV light (360 nm).

3.6.3.5. Gas-chromatography.

(J) Silylation. The hormonal compounds are silylated in the following manner. Evaporate a suitable extract amount containing 50 - 100 µg of the hormonal compound. Add 0.2 ml BSA (N,O-bistrimethylsilyl acetamide). Heat at 60°C for 10 minutes. Blow

TABLE 3-XVIII RESULTS OF THE BIOLOGICAL AND CHEMICAL ASSAY OF HORMONAL SUBSTANCES IN SELECTED COSMETIC MARKET SAMPLES (1973)

Claimed action	No	Product	Biological assay *	**	Chemical assay
Anti-wrinkle for older women.	1	face cream	a a a	E -	-
	2	face cream	a a a	E -	-
	3	throat cream	a a a	E -	-
	4	face cream	a a a	E -	-
	5	face cream	a a a	E -	-
	6	face cream	a a a	E -	-
	7	face cream	a a a	E -	-
	8	eye cream	a a a	E -	-
	9	face lotion	a a a	E -	-
	10	face cream	a a a	E -	-
	11	placenta cream	a a a	E -	-
	12	eye cream	a a a	E -	-
	13	night cream	a a a	E -	-
	14	night cream	a a a	E -	0.03% ethisteron
	15	face cream	a a a	E -	-
	16	face cream	a a a	E -	0.34% pregnenol-on acetate
	17	placenta cream	a a a	E -	-
	18	face cream	f b/d f	E +	0.0025% DES
	19	placenta cream	a a a	E -	-
	20	throat cream	a a a	E -	-
	21	décolleté cream	a a a	E -	-
	22	face cream	a a a	E -	-
	23	face cream	a a a	E -	-
	24	face cream	a a a	E -	-
	25	face cream	a a a	E -	-
	26	placenta cream	a a a	E -	-
	27	beautifying oil	a a a	E -	-
	28	face cream	a a a	E -	-
	29	face cream	a a a	E -	-
	30	royal yelly cream	a a a	E -	-
	31	placenta extract oil	a a a	E -	-
	32	idem aqueous	a a a	E -	-
	33	face cream	a a a	E -	-
	34	face mask	a a a	E -	-
	35	face cream	a a a	E -	-
	36	face cream	a a a	E -	-
	37	face cream	a a a	E -	0.23% pregnenol-on acetate
	38	face cream	a a a	E -	-
	39	face cream	a a a	E -	-
Development of the female breast.	40	bust cream	a a a	E -	-
	41	paper plaids	a a a	E -	-
	42	paper plaids	a a a	E -	-
Depression of female hair-growth	43	body cream	f f f	E +	0.1% DES
	44	body cream-mild	f f f	E +	0.01% DES
	45	body cream	a a a	E -	-
Promotion of male hairgrowth	46	anti-baldness		A -	-
	47	anti-baldness		A -	-
	48	moustache grower		A -	-

* Cytological evaluation (a=negative)(b/c=doubt)(d/e/f=distinct to very positive
** E - = Oestrogenic action absent ; A - = Androgenic action absent.
*** Quantitative biological and chemical assay on Table 3-XIX.

TABLE 3-XIX. COMPARISON OF THE QUANTITATIVE BIOLOGICAL AND CHEMICAL ASSAY OF A COSMETIC SAMPLE CONTAINING OESTROGENIC SUBSTANCES.

Biological assay of sample no.18

Sample equivalent in mg which has been injected subcutaneously as an extract in oil (0.3 ml)		Cytological evaluation (vaginal smear). See Table 3-XVIII under *	Oestrogenic activity
Sample no.18:	100 mg	f/d f f	E +
	40 mg	e/f e/f e/f	E +
	20 mg	f f f/d	E +
	10 mg	d/e d/e e	E +
	10 mg	e/f e e	E +
	5 mg**)	a/b f/d a/c	E ±**)
Standard	0.075 ug oestrone	e/f e/f e/f b/c c	E +
	0.15 ug oestrone	f e f f f e	E +

**) This is the limit of detection, which corresponds to 0.5 to 1 Mouse Units of oestrogenic activity (= 0.05 - 0.1 ug oestrone). Conclusion: 5 mg of sample no.18 contains 0.5-1 M.Units, 1 gram will contain 100 - 200 M.Units of oestrogenic activity (= equivalent to 10 - 20 ug oestrone per g)

Chemical assay of sample no.18

25 ug diethylstilboestrol per g.

off excess BSA. Dissolve in 250 µl CS_2. Inject ca. 3 µl.
(K) GLC conditions. Column 5% SE30 (or OV-17) on Chromosorb G. Dimensions 150 cm x 2 mm I.D. , glass. Temperature column for SE30: 190°C and for OV17: 230°C. Detector FID, 240°C. Carrier gas : nitrogen 30 ml per min.

3.6.4. Results of the biological and chemical assay of hormonal substances in the market survey.

The results of the biological and chemical assay of 48 market samples which were purchased in 1973-1974 are given in Table 3-XVIII. In practically all samples of anti-wrinkle preparations (including creams with placenta extracts) no detectable oestrogenic activity was found (except sample no 18) using the biological method. This fact was confirmed by chemical analysis. By chemical means two samples were shown to contain pregnenolone and one sample ethisterone. Neither of the steroidal compounds had any detectable oestrogenic activity, which supports the finding of earlier workers (Silson, 1962; Bullough et al., 1968). Sample no 18 was evaluated quantitatively by the quantitative biological method. Table 3-XIX gives the results. As can be seen the biological data (100-200 Mouse Units per g, which corresponds to 0.0010-0.0020% oestron equivalents) were in fair agrement with the chemical findings (0.0025% DES), as the biological activity of DES when administered subcutaneously in oil solution, was shown to be approximately equal to that of oestrone (Huis in't Veld, unpublished data). The three samples for promotion of female breast development (no 40,41,42) lacked detectable oestrogenic activity. Of three samples for depression of abnormal growth of hair in the female (no 43,44,45), two showed considerable oestrogenic activity (no 43,44, both of the same brand) which has been confirmed by the results of chemical analysis. The anti-baldness preparations and moustache-growers (no 46,47,48) did not show androgenic activity. The absence of testosterone by chemical means confirms these findings.

3.7. Antimicrobial compounds

Antiseptics and preservatives belong to the same class of potentially risk-bearing compounds, namely "antimicrobials". Next to perfume components antimicrobials are frequently the cause of adverse skin reactions. It is therefore of the utmost importance to study the identification and determination of this important class of compounds.

A systematic search for antimicrobials in cosmetics, starts with TLC, which is

TABLE 3-XX INVENTORY , CLASSIFICATION , CONCENTRATION-LEVELS OF ANTIMICROBIALS IN COSMETICS.

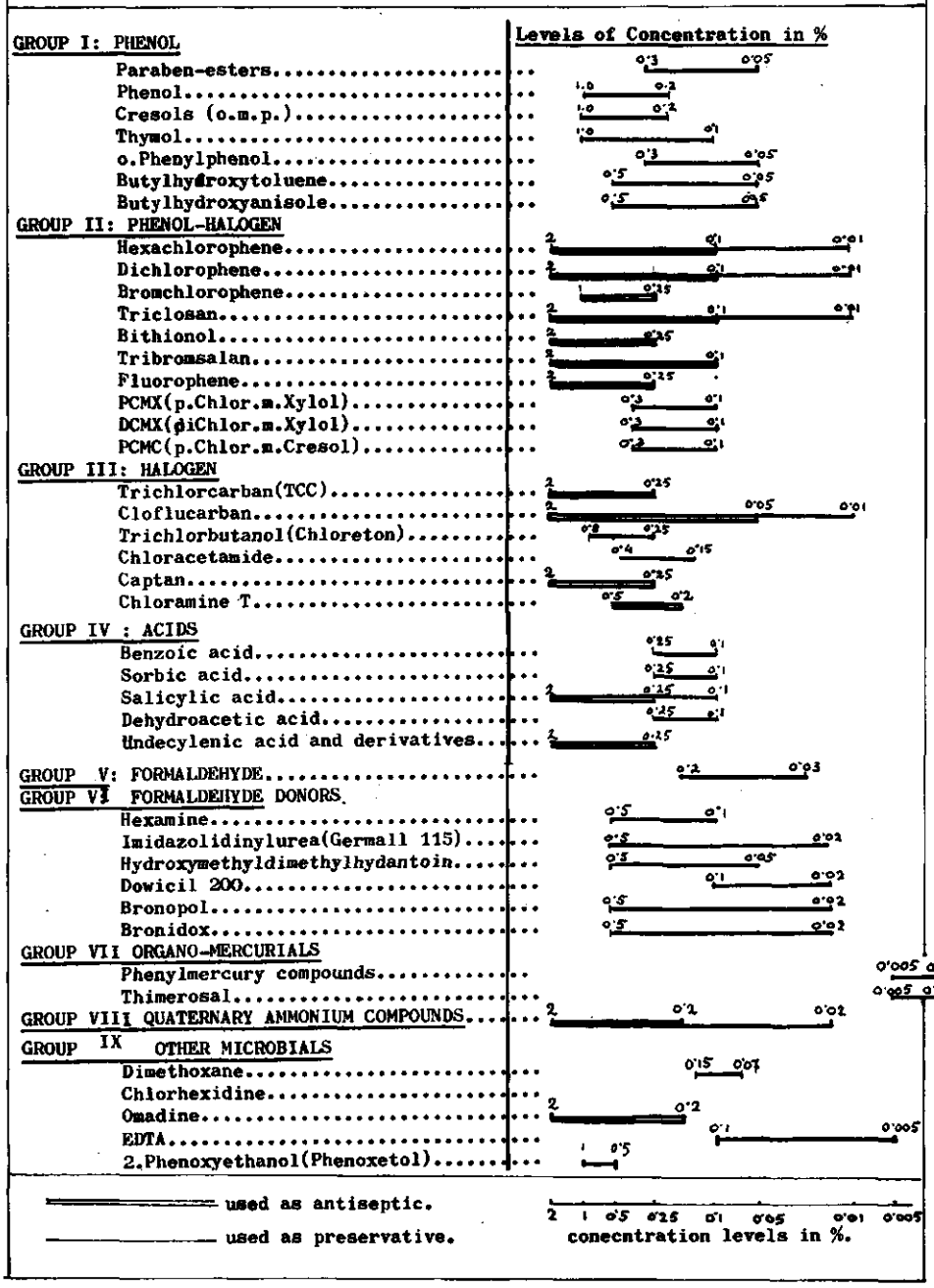


TABLE 3-XXI FORMULAE OF ANTIMICROBIALS (1)

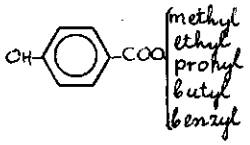
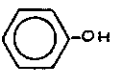
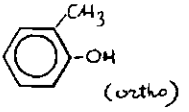
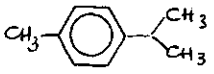
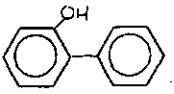
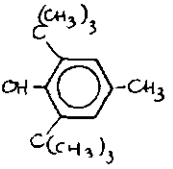
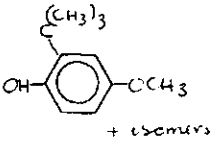
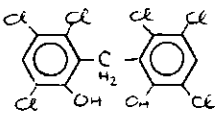
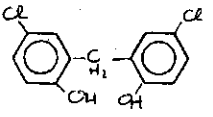
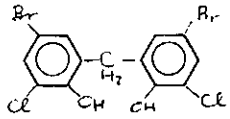
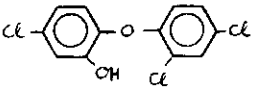
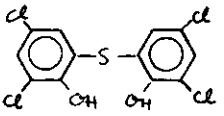
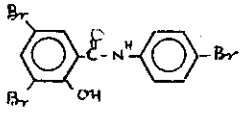
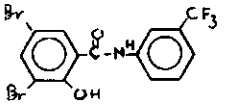
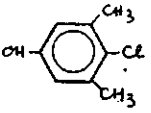
Gr. I  **Paraben-esters**	Gr. I  **Phenol**	Gr. I  **Cresol (o.m.p.)**
Gr. I  **Thymol**	Gr. I  **o.Phenylphenol**	Gr. I  **Butylhydroxytoluene (BHT)**
Gr. I  **Butylhydroxyanisole (BHA)**	Gr. II  **Hexachlorophene**	Gr. II  **Dichlorophene**
Gr. II  **Bromochlorophene**	Gr.II  **Triclosan**	Gr.II  **Bithionol**
Gr.II  **Tribromsalan**	Gr. II  **Fluorophene**	Gr. II  **PCMX (p.Chlor.m.xylol)**

TABLE 3-XXI FORMULAE OF ANTIMICROBIALS (2)

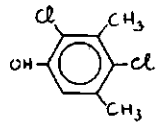
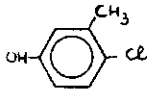
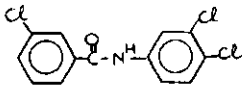
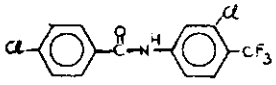
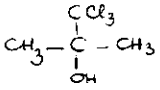
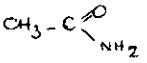
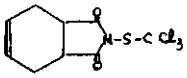
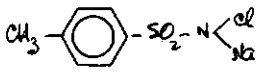
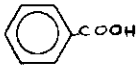
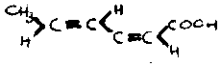
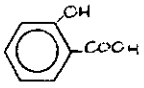
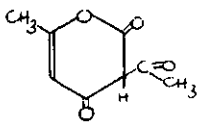
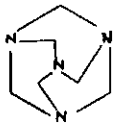
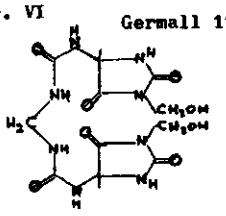
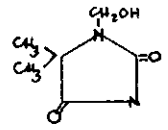
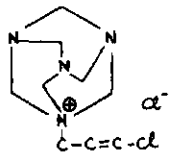
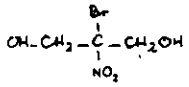
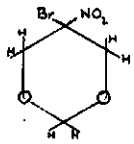
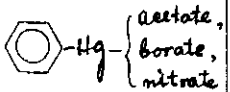
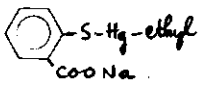
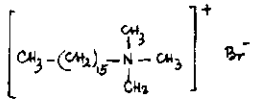
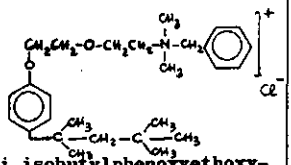
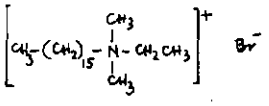
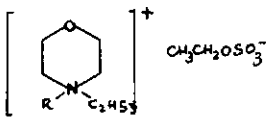
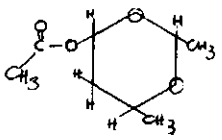
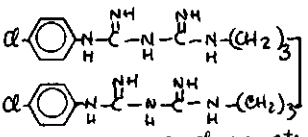
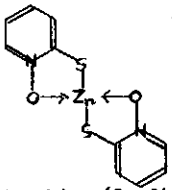
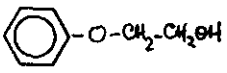
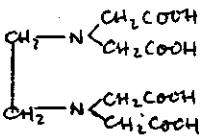
Gr. II  DCMX (di.Chlor.m.xylol)	Gr. II  PCMC (p.Chlor.m.cresol)	Gr. III  Trichlorcarban (TCC)
Gr. III  Cloflucarban (IrgasanCF3)	Gr. III  (Chloreton) Trichlorbutanol	Gr. III  Chloracetamide
Gr. III  Captan	Gr. III  Chloramine T	Gr. IV  Benzoic acid
Gr. IV  Sorbic acid	Gr. IV  Salicylic acid	Gr. IV  Dehydroacetic acid (DHA)
Gr. V  Formaldehyde	Gr. VI  Hexamine	Gr. VI Germall 115  Imidazolidinyl urea

TABLE 3-XXI

FORMULAE OF ANTIMICROBIALS (3, final).

Gr. VI  Hydroxymethyldimethyl-hydantoine	Gr. VI  Dowicil 200	Gr. VI  Bronopol
Gr. VI  Bronidox	Gr. VII  Phenylmercury salts	Gr. VII.  Thimerosal (Merthiolate)
Gr. VIII Cetavlon  Cetyltrimethylammonium bromide	Gr. VIII Hyamine1622  Di.isobutylphenoxyethoxy-ethyl.dimethyl.benzyl-ammonium chloride	Gr. VIII Bretol  Cetyl.dimethyl.ethyl-ammonium bromide .
Gr. VIII Quaternium 2  ium ethosulphate N-soya.N-ethyl morpholin-	Gr. IX  Dimethoxane	Gr. IX  as gluconate, acetate, chloride. Chlorhexidine
Gr. IX  Pyridinethion (Omadine)	Gr. IX  2.Phenoxyethanol	Gr. IX  EDTA

described in detail in section 3.7.2.1. Not all groups, however, can be detected by TLC. Formaldehyde , organo-mercurials, quaternary ammonium compounds, EDTA should be analysed separately by the methods described in section 3.7.2.

3.7.1. Inventory, classification and use of antimicrobials in cosmetics.

Based on technological information from chemical supply houses and from cosmetic manufacturers, a choice of approximately 50 antimicrobials has been made to start the analytical study. The result is presented in Table 3-XX. This table also gives a classification of the selected compounds and expected levels of concentration have also been studied to facilitate the analysis. These levels of concentration generally run parallel to the intended cosmetic action. Preservation is achieved at levels of 0.01-0.25%. For powerful antimicrobial compouds this level is even lower. Organo-mercurials are used at levels of 0.002-0.007% and chlorhexidine at approx. 0.005%. Combinations of preservatives are often used because of expected synergism or broadening of the antimicrobial spectrum. Lower levels can then be used, which will also lower the incidence of skin reactions. If an antiseptic action is aimed at, as in disinfectant soaps, levels of 1-2% of the antimicrobial compound can be expected. Sometimes a mild antiseptic action is aimed at which can result in a deodorizing effect. Levels of 0.1-1,0% -intermediate between the extremes - can then be expected.

3.7.2. Analytical methods.

Several papers have been published which deal with the analysis of the different classes of antimicrobial compounds in cosmetics (Graber et al.,1969; König, 1969,1973; Karleskind et al., 1972; Wilson, 1975). Most workers use chromatographic methods to identify and to determine these antimicrobial compounds.

3.7.2.1. Detection and preliminary information.

Much information can be obtained from TLC analysis withim a short time, especially for the first groups of compounds given in Table 3-XX.

It is important to apply optimal amounts on a TLC-plate. Too small amounts of extracts might give false negative results. Optimal amounts can only be found by trial and error. Information of expected concentration levels from Table 3-XX will be also helpful in the analysis.

(A) Extraction with methanol (for products with a high oil content). Mix in a small 10 ml bottle with screw cap, 1 g product + 0.25 ml HCl 4M + 0.5 ml paraffine oil

(liquifying aid for solid lipids)+ 5 ml methanol. Homogenize by slightly warming. Shake. Add a little anh.sodium sulphate. Shake. Let mixture settle. Use clear layer for TLC or GLC.

(B) Extraction with benzene (for products with low oil content). Remove organic solvents (ethanol etc.) by heating a weighed amount (1 g) on a waterbath. It is not necessary to remove the water. Then add 0.25 ml HCl 4M and 5 ml benzene. Shake and homogenize. Add a little anh.sodium sulphate to bind water. Shake. Let mixture settle. Use clear layer for TLC or GLC.

(C) Solvent systems for TLC on precoated silicasheets with fluorescent indicator.

S1 = toluene-acetic acid (80-20, by vol.)

S2 = n.hexane-ethylacetate-acetic acid (80-10-10, by vol.)

The Rf's of most of the selected antimicrobial compounds are given in Figure 3-Q.

Visualization (see Table 3-XXVI) can be made by the following four methods.

V1 = UV light of 254 nm. Dark spots of many compounds will be visible.

V2 = Kovacs spray with silver nitrate, and using Stijve & Cardinale's method(1974). Spots of 1 µg of halogenated antimicrobial compounds are clearly visible. Dissolve 0.5 g silver nitrate in 1 ml water and dilute to 100 ml with ethanol. Keep cool and in a dark place. Spray abundantly on TLC plate. Wait 10 minutes. If spots appear within that time they are not from organic-halogen. Expose for 10 minutes under short-wave UV light. Use a light source of sufficient intensity (for instance Philips TUV, 15 watt) and at a distance of 15-20 cm. Spray with water. Expose again under UV light. After 1-2 minutes black spots will appear.

V3 = Gibb's spray for phenolic compounds. Sensitivity to 5 µg spots. Reagent I: Prepare a fresh solution of 0.4% 2.6.dibromquinonchlorimide in methanol. ReagentII: A solution of 10% sodium carbonate in water. Use: Spray succesively with I and II. Optimal blue to violet colours appear after 10 minutes.

V4 = Millon's reagent (Hg II - nitrate)is the best reagent to visualize parabens, which do not react with Gibb's reagent (in spite of the free phenolic group). Because of the toxicity of the reagent, the following procedure was developed in our laboratory. Use Merck reagent 9026. Cut a piece of filter paper and dip into the reagent. Place this paper right across the TLC plate at the zone of the parabens as detected under UV light. Press the paper on to the TLC plate by means of a glass plate. After several minutes, if necessary by warming slightly, the parabens will give pink-red spots (methyl,ethyl,propyl ester give red spots; butyl,benzyl ester give pink spots). Phenoxetol (2.phenoxyethanol of group IX), which is often combined with the parabens gives a yellow colour.

TLC detection should always be considered as a first step of identification. In particular the following seven compounds with close Rf's of solvent S1, need further confirmation , for instance by GLC.

TABLE 3-XXII TLC OF ANTIMICROBIALS : VISUALIZATION TABLE.

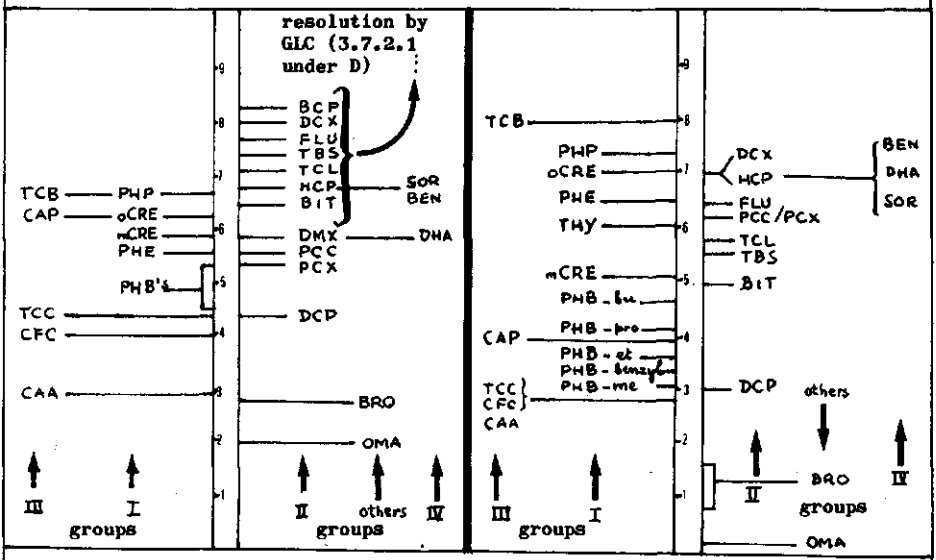
1.*	2.*	3.*	4.*	5.*	6.*	7.*
PHB	(I)	Paraben esters	++++	-	-	pink-red
PHE	(I)	Phenol	-	-	+ blue	yellow-brown
CRE	(I)	Cresols (o.m.p.)	-	-	+ purple-blue	yellow
THY	(I)	Thymol	-	-	+ violet-blue	
PHP	(I)	o.Phenylphenol	+	-	+ blue	brown
BHT	(I)	Butylhydroxytoluene	-	-	+ violet	
BHA	(I)	Butylhydroxyanisole	-	-	+ violet-blue	
BNT	(I)	beta-Naphthol	-	-	+ dark brown	yellow
HRS	(I)	Hexylresorcinol	+	-	+ brown	yellow
HCP	(II)	Hexachlorophene	+	++++	+ light blue	
DCP	(II)	Dichlorophene	+	+++	+ blue	
BCP	(II)	Bromochlorophene	+	+++	+ deep blue	
TCL	(II)	Triclosan	+	++++	+ blue	
BIT	(II)	Bithionol	++	+++	+ blue	
TBS	(II)	Tribromsalan	++	+	+ slowly lightblue	
FLU	(II)	Fluorophene	++	+	+ starts pink, change to light blue	
PCX	(II)	PCMX(p.chlor.m.xylol)	-	++++	+ dark blue	
DCX	(II)	DCMX(dichlor.m.xylol)	-	++++	+ blue	
PCC	(II)	PCMC(p.chlor.m.cresol)	-	++++	+ blue	
TCC	(III)	Trichlorcarbanilide	+++++	++	-	
CFC	(III)	Cloflucarban	++	+++	-	
TCB	(III)	Trichlorbutanol	-	+++++	-	
CAA	(III)	Chloracetamide	-	+++	-	
CAP	(III)	Captan	-	+	-	
BEN	(IV)	Benzoic acid	+	-	-	
SOR	(IV)	Sorbic acid	++++	-	-	
DHA	(IV)	Dehydroacetic acid	+	-	-	
BRO	(VI)	Bronopol	-	++	-	
CHX	(IX)	Chlorohexidine	-	++	-	
OMA	(IX)	Omadine	-	-	+ violet	
PHX	(IX)	Phenoxetol	-	-	-	yellow

* 1 Abbreviations used in this report.
2. Group classification according to Table 3-XX.
3. Name of the antimicrobial compound.
4. Fluorescence quenching under UV-light (dark spots). Spots of 10 µg.
5. Kovacs silver nitrate spray (3.7.2.1 under C). Spots of 1 µg.
6. Gibb's spray for phenolic groups (3.7.2.1 under C) Spots of 5 µg.
7. Millon's reagent (Hg^{II} nitrate) (3.7.2.1 under C) Spots of 10 µg.

FIGURE 3-Q TLC OF ANTIMICROBIALS: Rf-SCHEME

Solvent system S1
toluene-acetic acid (80-20)

Solvent system S2
n.hexane-ethylacetate-acetic acid (80-10-10)



Abbreviations: (roman ciphers refer to group classification of Table 3-XX)

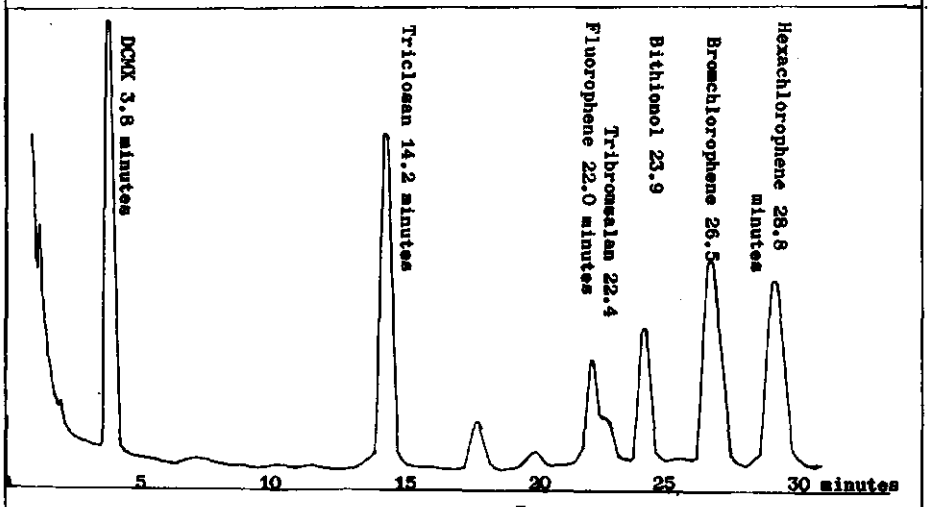
BEN	benzoic acid (IV)	DMX	dimethoxane (IX)
BCP	bromochlorophene (II)	FLU	fluorophene (II)
BIT	bithionol (II)	HCP	hexachlorophene (II)
BRO	bronopol (VI)	OMA	omadine (IX)
CAA	chloracetamide (III)	PCC	p.chloro.m.cresol (II)
CAP	captan (III)	PCX	p.chloro.m.xylol (II)
CFC	cloflucarban	PHB	p.hydroxy.benzoic esters (I)
oCRE	o.cresol (I)	PHP	o.phenylphenol (I)
mCRE	m.cresol (I)	SOR	sorbic acid (IV)
DCP	dichlorophene (II)	TBS	tribromsalan (II)
DCX	dichloro.m.xylol (II)	TCL	triclosan (II)
DHA	dehydroacetic acid (IV)	THY	thymol (I)

(D) GLC confirmation procedure for seven compounds with close Rf, namely hexachlorophene, bromochlorophene, bithionol, DCMX, triclosan, tribromsalan, fluorophene. Purify by TLC as described under section 3.7.2.2. an extract amount containing ca. 100 µg of the active compound. Use S1 as solvent. Elute the compound by shaking the silica with 0.5 ml benzene containing 0.1% dicyclohexylphthalate as an internal standard. Use micro glass filter (for example Jena 25.8575.3- 2 ml) to obtain clear solution. Mix 100 µl of clear filtrate with 100 µl BSA (N,O bis trimethylsilyl acetamide). Inject 5 µl of the mixture in the gas-chromatograph. A good resolution of the seven compounds can be achieved. See Figure 3-R
(E) GLC conditions. Column 1.5 m x 4 mm i.d., filled with 5% UCW 982 on Chromosorb G. Temp.inj.port 230°C. Detector FID. Temperature column 130°-230°C (4°per min)

Formaldehyde, an important preservative of cosmetics, is not detected by chromatographic methods, but more easily by colour reactions. A very sensitive reaction , described by Harkins et al.(1974), was modified and adapted for the detection of formaldehyde in all kinds of cosmetic products. The procedure is:
(F)The reagent is a 1% solution of 4.amino-3.hydrazino-5.mercapto-1.2.4.triazole (AHMT = Purpald of Aldrich Chemicals = Merck no 10787) in NaOH 1M. Divide the product into a transparent layer on a glass plate with white paper as a background. Apply 1 drop of the reagent on the sample. A violet-red colour will appear within 3 minutes. The chemical reaction of this colour formation is shown on Figure 3-V. Even coloured cosmetics can be analysed by this procedure. But unfortunately many other aldehydes also give a positive reaction (for instance dimethoxane, an acetaldehyde donor compound; glutardialdehyde, which is sometimes used as a disinfectant in cosmetic plants; perfume components). Formaldehyde donors (group VI) will also give a positive reaction, due to the presence of "free"formaldehyde. The following lowest concentrations, which will still give a clear positive violet-red colour, were found: 0.0010% formaldehyde ; 0.0050% Bronopol ; 0.0025% Dowicil 200 ; 0.0250% Germall 115 ; 0.0025% hexamine ; 0.0100% MDH hydantoin ; 0.0050% dimethoxane).

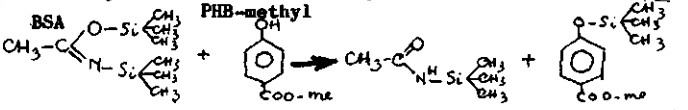
Identification of formaldehyde donors can be made by TLC, as reported by Ryder (1974) and Wilson (1975). The following procedure was used, consisting of a solvent system of Ryder and a visualization method of Wilson:
(G) Use silicasheets without a fluorescent indicator. Solvent system: ethylacetate-methanol-ammonia (65-30-5, by vol.). Extract 1 g sample with a mixture of 1.5 ml acetone+2 ml methanol+1.5 ml water. Centrifuge. Use amount extract containing ca. 40 µg formaldehyde donor compound per spot. Dry developed plates for 5 minutes at 80°C. Spray with Hantzsch reagent (15.4 g amm.acetate+0.2ml acetylacetone+ 0.3ml acetic acid; dissolve in 100 ml water). Dry on hot plate for 5 min. at 50°C. View under UV light for fluorescent spots. The following Rf were found: hexamine 0.34 ; Bronopol 0.86 ; Dowicil 200 0.62 ; Germall 115 with two spots 0.11 and 0.16.

FIGURE-3R GLC CONFIRMATION OF THE TLC-IDENTIFIED PHENOLIC COMPOUND.



GLC conditions according to 3.7.2.1 under E.

Example of the silylation reaction with N,O bis-trimethylsilyl acetamide (BSA):



Examples for retention times of the silylated derivatives:

	Column temp.			Column temp
Methyl-paraben	3.4 min at	130°C	Hexachlorophene	9.2 min at 230°C
Ethyl-paraben	5.3 min	130°C	Dichlorophene	3.4 min 220°C
Propyl-paraben	7.8 min	130°C	Bromochlorophene	11.0 min 220°C
Butyl-paraben	6.8 min	145°C	Triclosan	2.3 min 220°C
Benzyl-paraben	7.8 min	180°C	Bithionol	7.4 min 220°C
Paraben-acid	7.2 min	130°C	Tribromsalan	5.4 min 230°C
o.Phenylphenol	2.0 min	100°C	Fluorophene	3.0 min 220°C
o.Cresol	1.3 min	100°C	PCMX	3.8 min 120°C
p.Cresol	1.9 min	100°C	DCMX	5.1 min 130°C
m.Cresol	1.8 min	100°C	PCMC	2.2 min 120°C

Examples for retention times of internal standard compounds:

Dicyclohexylphthalate	7.7 min	210°C	Benzophenone	3.4 min 145°C

The optimal amounts for TLC spots for Dowicil 200 was 1 µg, and for all the other donor compounds 5-10 µg .

Quaternary ammonium compounds (group VIII) in cosmetics are easily detected in cosmetics by its pink to brown colour of their permanganate complexes. Procedure (D) of section 3.7.2.4. describes the analysis.

Organo-mercurial compounds, which are used at very low levels of concentration in cosmetics (0.0030-0.0070%) cannot be detected by simple and fast analytical procedures. Destruction to inorganic mercury and detection by atomic absorption spectroscopy (AAS) is the only way to detect the mercurial compounds. Further details are described in section 3.7.2.4.

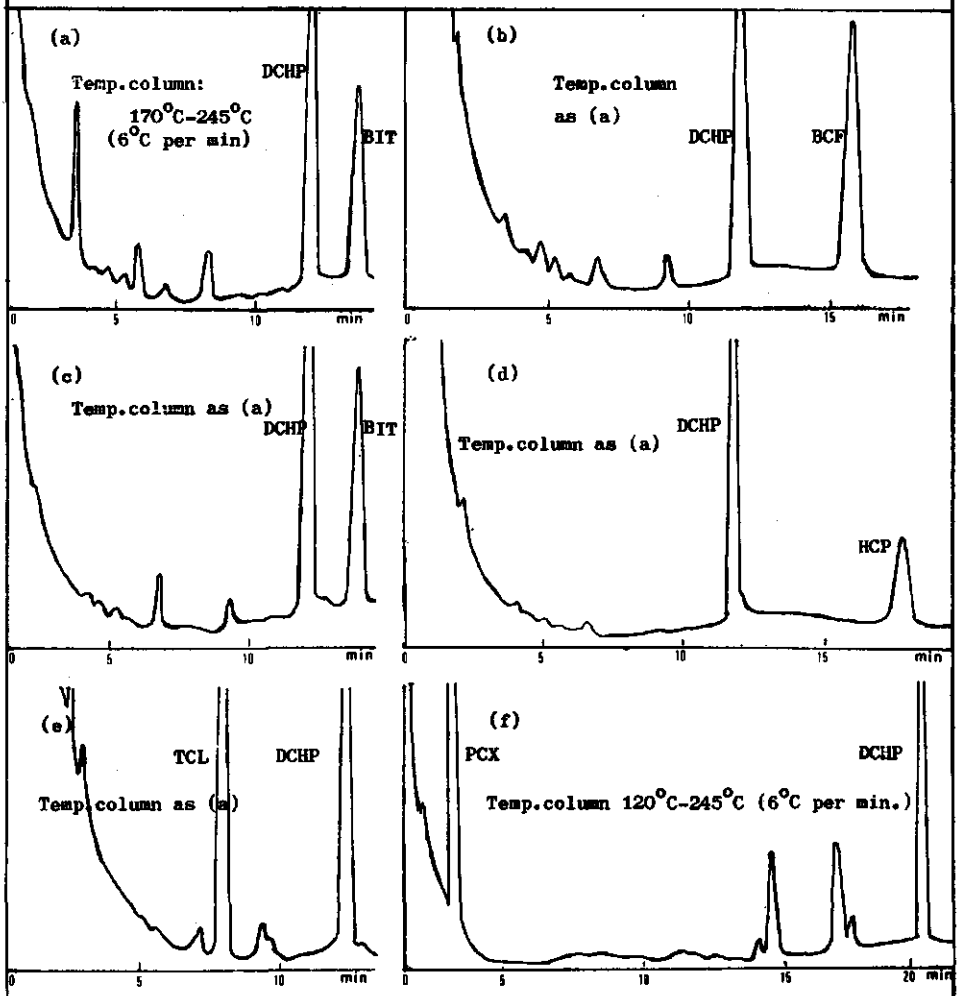
3.7.2.2. TLC clean-up procedure.

In quantitative determinations of compounds, which are used in many kinds of formulations, TLC is a powerful tool for a clean-up procedure. In section 3.4.5. the UV-determination of sunscreens in suntan products after a TLC clean-up procedure, on ordinary commercial TLC-sheets was described. In this section which concerned antimicrobials, the TLC clean-up procedure was again succesfully employed, prior to GLC determinations. As an example hexachlorophene, an antibacterial compound, which can be used in many cosmetic products, such as milks, creams, shampoos, eye cosmetics, aerosols, powders, soaps etc. is mentioned. Conventional solvent partitioning or column chromatography is used to purify the extracts. A TLC clean-up procedure, however, is much more simple and faster in operation. It proceeds automatically and one needs only a chromatographic tank and commercially available TLC sheets. It takes 30-40 minutes and the results of the clean-up process can be easily seen and judged under UV-light. If the results are not satisfactory another solvent system can be used.

In studying TLC conditions for obtaining quantitative results the de-activation of commercial TLC silica sheets was found to be the most important operation. It prevents losses of the compound which might be due to irreversible adsorption of the silica support.

Experimental: De-activate commercial pre-coated silica sheets (with fluorescent indicator) by spraying with water on the silica until glazed. Dry by leaving the wet sheets at room temperature during ca. 3 hours. Apply extract on starting line over a distance of 4-8 cm. Reference standard solutions can be run on the same plate. If necessary control strips - for aids of localization - are also run on both sides of the extract. Localize, after development, by viewing under UV-light or by spraying the control strips with specific sprays (e.g. Gibb'spray for phenolic compounds; see 3.7.2.1. under procedure C). The localized zone of the antimicrobial compound is cut off with scissors and the silica support carefully scraped off.

FIGURE 3-S EFFECT OF TLC-CLEANUP ON THE GLC PATTERNS OF SILYLATED "HALOGENATED PHENOLS" EXTRACTED FROM COSMETIC SAMPLES.



GLC conditions according to 3.7.2.1. under E., determination according 3.7.2.3.

(a) Protein shampoo spiked with bithionol. Recovery yield: 98.3%.

(b) Dentifrice spiked with bromchlorophene. Recovery yield: 96.9%.

(c) Eye-liner spiked with bithionol. Recovery yield 91.9%.

(d) Foot-lotion spray containing hexachlorophene 0.10%.

(e) Deodorant spray containing 0.10% triclosan.

(f) Mascara spiked with PCMX. (Column temp. was programmed from 120°- 245°C, 6°C per min.)

DCHP (dicyclohexylphthalate) was used as an internal standard.

Extract the compound with a suitable solvent, in which a known amount of an internal standard compound can be added. Addition of such an internal standard has the great advantage that filtration or evaporation losses do not affect the final results.

The effectiveness of the described TLC clean-up procedure is shown on Figure 3-S . The figure shows GLC-patterns of the purified extracts of many different kinds of cosmetic products.

3.7.2.3. Determination of phenols and halogenated phenols.

The groups I and II of the antimicrobial compounds have very important members, such as the paraben preservatives (p.hydroxybenzoic esters) and the chlorophene bactericides (hexachlorophene, dichlorophene, bromochlorophene). These compounds have a phenolic group common, and because of this they can be determined by a same basic procedure, namely by gaschromatography after derivatization.

As a first example the determination of the paraben esters will be described. Extract the parabens by methanol as described in section 3.7.2.1 under (A). Purify by TLC according to 3.7.2.2. and using the S2 solvent (3.7.2.1) mixture n.hexane-ethylacetate-acetic acid (80-10-10, by vol.) an amount extract containing 50-100 µg of each of the paraben esters. Use an ethereal solution of benzophenone (internal standard) 0.01% as extracting solvent for the silica. Only 0.5 ml is needed per extraction. Leave the mixture to settle. Mix 200 µl of the clear layer with 100 µl of BSA (N,O bis.trimethylsilyl acetamide. Inject 1-2 µl of the mixture in the gas-chromatograph. Silylation will take place in the injection port ("flash silylation"). GLC conditions: Column made of glass, 1.5 m X 4 mm i.d., filled with 5% OV-17 on Chromosorb WAW-DMCS; injection port 230°C; FID; column temperature for

methyl-propyl paraben: 130°C isotherm.

methyl-ethyl-propyl-benzyl: 8 min at 130°C, progr. 10°C per min to 250°C and 8 min at 250°C.

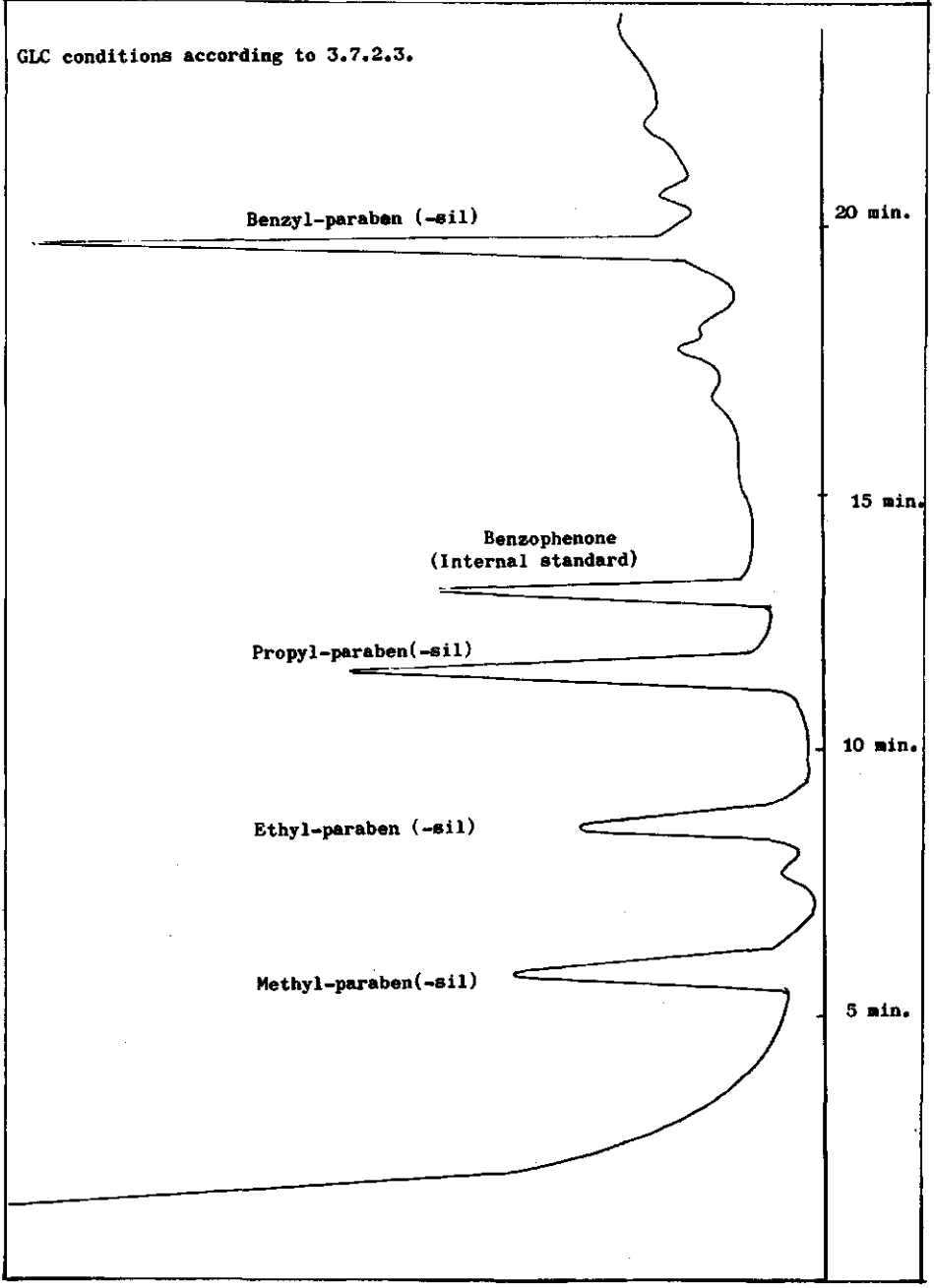
methyl-ethyl-propyl-butyl : same as for previous mixture, except only up to 200°C.

Figure 3-T is an example of this GLC separation of the silylated paraben-esters. In quantitative determinations the use of a standard curve is recommended. The following standard solutions must be prepared: Solution (I) of the internal standard = 10 mg benzophenone dissolved in 100 ml ether; solution (S) = 100 mg of a paraben ester dissolved in 100 ml ether.

S1 = mixture of 1 ml (I) + 50 µl (S) (contains 25 µg paraben ester per 0.5 ml)
S2 = mixture of 1 ml (I) + 100 µl (S) (contains 50 µg paraben ester per 0.5 ml)
S3 = mixture of 1 ml (I) + 150 µl (S) (contains 75 µg paraben ester per 0.5 ml)
S4 = mixture of 1 ml (I) + 200 µl (S) (contains 100µg paraben ester per 0.5 ml)

Inject 1-2 µl of these standard mixtures into the gas-chromatograph. Plot on a graph: X-axis the amount (µg) paraben ester per 0.5 ml and for the Y-axis

FIGURE 3-T GLC-SEPARATION OF SILYLATED PARABEN-ESTERS.

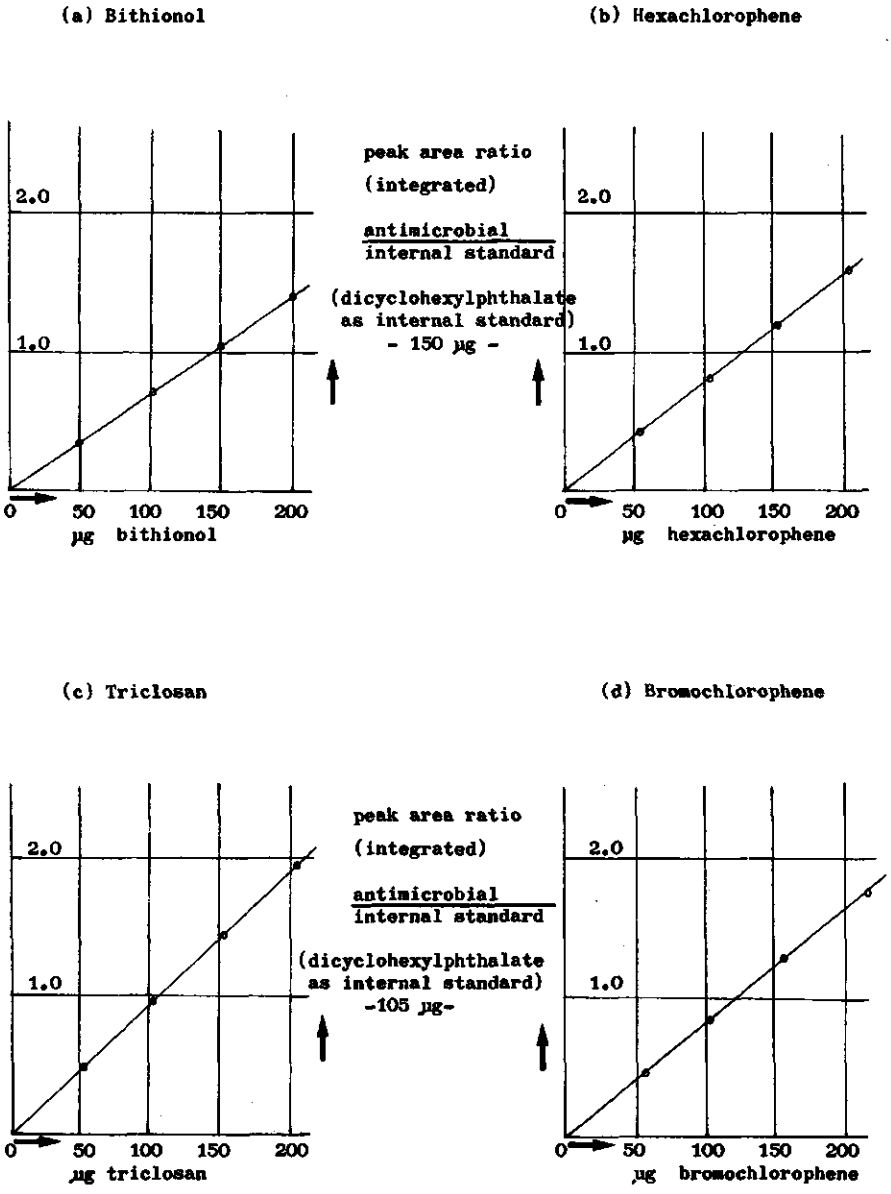


the peak area ratio paraben ester/ benzophenone. Recovery tests for the TLC clean-up of the ethyl ester have been made with the following results: 97%-102%-96%-95%. The "flash silylation" procedure has also been tested for reproducibility by making 7 succesive injections of freshly prepared mixtures of an ethyl-paraben solution and the silylating agent (BSA). The peak heights obtained were: 13.8-13.7-13.1-13.3-13.4-12.8-13.5 mm. The mean was 13.4mm $\pm$ 0.3%.

A second example is the determination of triclosan. Triclosan is extracted with benzene (according to 3.7.2.1.under B). Purify by TLC an extract amount containing 50-100 µg triclosan and using the solvent mixture S1 (toluene-acetic acid 80-20) to develop. Localize triclosan zone under UV-light and extract triclosan from silica with 0.5 ml 0.1% dicyclohexylphthalate in benzene. Shake and leave mixture to settle. Use ca.0.3 ml of clear layer. Remove solvent by blowing with air and without heating. Add 100 µl BSA (N,O bis-trimethylsilyl acetamide). Heat at 60°C for 10 min.in closed vessel. Cool. Add 200 µl CS_2. Mix. Inject 2-4 µl in the gas-chromatograph. Calculate peak area ratio (triclosan/DCHP = r_{sm}). Silylate by the same procedure 100 µl 0.1% triclosan in benzene. Peak area ratio = r_{st}. The % triclosan is calculated by the formula: $(r_{sm}/r_{st}).(50/a.w)$, in which w = g sample in 5 ml extract; a = µl extract used for TLC clean-up. The GLC conditions are: glass column, 1.5 m X 4 mm i.d., filled with 5% UCW 982 or SE30 on Chromosorb G. Temp.inj.port 230°C. FID. Column temp.220°C. The retention time for triclosan is ca. 2.3 min. and for DCHP 6 minutes. The linearity of the detector response is shown on Figure 3-U. The described method for triclosan can be used for hexachlorophene, bromochlorophene and for bithionol. See Figure 3-U. However, it is important to note that bithionol decomposes under UV-light. Localize bithionol not under UV-light, but only by means of Gibb's spray (section 3.7.2.1)on control strips.

The methods described above are suitable for the determination of phenolic compounds at concentration-levels of 0.1-1.0%. Lower levels can be determined by gas-chromatography of the methylated compound and using an electron-capture detection. Ernst et al.(1974)determined hexachlorophene by this method. Their procedure was combined with our TLC purification method and methylation of the purified hexachlorophene in a micro-apparatus designed by Fales et al.(1973). Inhalation and explosion hazards of the methylating agent diazomethane are practically eliminated by the use of this vessel. Diazomethane is generated in situ by the reaction of 2mMole (=200 mg) of N.methyl-N.nitroso-N'nitroguanidine (MNNG) and 1 ml NaOH 5M. Avoid, however skin contact with MNNG, as this substance is very strongly mutagenic. Methylation with diazomethane is instantaneous. As an internal standard methoxychlor can be used. Quantitative yields were obtained in recovery trials of hexachlorophene in spiked shampoos and creams, using the proposed TLC cleanup procedure, methylation in the micro-vessel of Fales et al., and GLC of the methylated compound with electron capture detection.

FIGURE 3-U LINEARITY OF STANDARD CURVES OF THE SILYLATED HALOGENATED PHENOLS.



3.7.2.4. Determination of the other antimicrobial compounds.

The halogen-containing antimicrobial compounds of group III were determined semi-quantitatively by visual comparison of TLC-spots, using the solvent system toluene-acetic acid (80-20,by vol.). Optimal spots of these compounds were obtained in the range of 0.5 - 2 µg, when using Kovacs spray (section 3.7.2.1). The separation of trichlorcarban (TCC) and cloflucarban (Irgasan CF3), however, was not very good. This method has been described by König (1969).

The acidic antimicrobial compounds of group IV were mainly determined by direct gaschromatography (without derivatization). It was necessary to saturate the carrier gas with formic acid before entering the injection port, in order to reduce tailing of these highly polar compounds. Saturation with formic acid was simply done by bubbling the carrier gas through a concentrated solution of formic acid. Care should be taken since only glass or stainless steel can withstand the corrosive action of formic acid. Details of this technique has been described by Ackman & Burgher(1963).

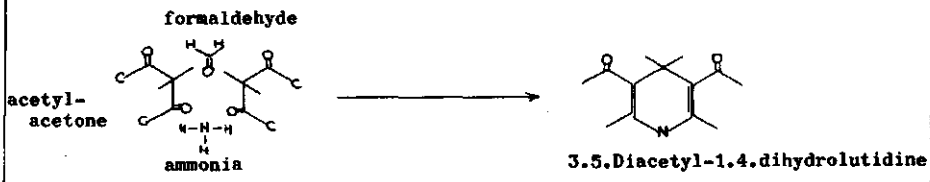
Experimental: Column 1.5 m X 2 mm i.d., glass, filled with 12.5% PPGS (polypropyleneglycolsebacate) on Chromosorb DMCS 80/100. Injection port: 250°C. Detector FID. Temperature of the column for benzoic acid: 190°C; sorbic acid 190°C; dehydroacetic acid 170°C. Pelargonic acid can be used as an internal standard.

Formaldehyde (group V)in cosmetics is most conveniently determined by the fluorometric method of Wilson (1974). The reaction (see Figure 3-V) is a condensation of formaldehyde, ammonia and acetylacetone to 3.5.diacetyl-1.4.dihydrolutidine, which can be measured fluorometrically. The reaction proceeds quantitatively after 1 h at 37°C. The lutidine derivative is excited at 415 nm and the resultant fluorescence emitted and measured at 505 nm. The necessity for pre-isolation of the formaldehyde is a matter of discussion. Pre-isolation of formaldehyde by microdiffusion, prior to the actual determination, has been proposed recently in the cosmetic study group of the Commission of European Communities (CEC, 1975). In the proposed method microdiffusion is done in glass Conway-cells and by warming for 18 h at 50°C. One should bear in mind that this microdiffusion procedure will not isolate formaldehyde quantitatively from the sample, but only for a part. After 18 hours warming at 50°C, a dynamic, but reproducible, equilibrium state must be established within the Conway cell. We have found that experimentally approximately 30-40% of the potentially available formaldehyde has been moved to the inner compartment (filled with water) of the cell. It is necessary to run parallel samples of standard solutions of formaldehyde in order to eliminate slight differences in equilibrium conditions during the determination.

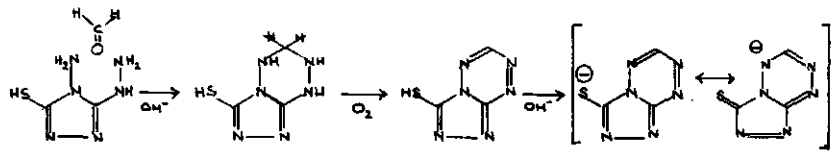
Obviously pre-isolation of formaldehyde by microdiffusion gives more assurance

FIGURE 3-V CHEMICAL REACTIONS IN THE ANALYSIS OF FORMALDEHYDE.

(a) Reaction in the fluorometric determination of formaldehyde. (Wilson,1974)



(b) A sensitive colour reaction for aldehydes in general and formaldehyde in particular. (Harkin et al.,1974)



4.Amino.3.hydrazino.5 mercapto.1.2.4.triazole (AHMT)

violet-red

= Purpald (Aldrich)

= 10787 (Merck)

in the determination, but in practice Wilson's direct dilution method has given very satisfactorily results with all kinds of cosmetic samples. Two difficulties, however, have to be considered. Creams and milks for instance might give turbid solutions after dilution with water and which are not suitable for the fluorometric determination. A clarification procedure has been introduced by Vaessen (1975) by the addition of Carrez solution during the dilution step. A second difficulty might arise with samples containing fluorescent dyes, e.g. bubble baths coloured with xanthene dyes. In such samples a correction has been introduced and which is obtained by the fluorescence measured in the diluted sample without the addition of acetylacetone.

Formaldehyde in cosmetic samples can also be determined by polarography and in many instances without prior isolation. Elving et al. (1948) have determined glycerol in fermentation residues by oxidation to formaldehyde and polarographic determination of this oxydation product. The method has been adapted here for cosmetic samples, which can simply be diluted with water to a level of 5-20 ppm. Clarification of turbid solutions as is necessary in the fluorometric determination, can be omitted in polarographic determinations. Some samples, however, might give distortions of the polarograhic wave. In such a case another method must be used.

(A) Polarographic determination (differential pulse) of formaldehyde in cosmetics.

Apparatus: Bruker E 100 polarograph, or any other instrument suitable for differential pulse polarography. Amplitude 30mV. Sweep 5mV/sec. Scan 1.5-2.0 Volt. Dropping mercury electrode. Dropping time 1 sec. Temp. 25°C. i=2μA. Gain 1 or 2.

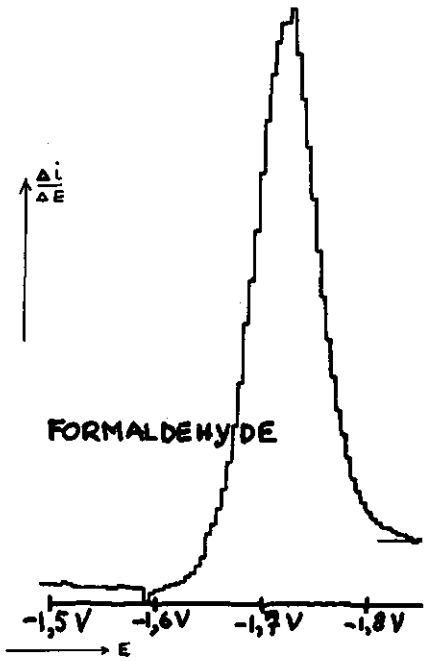
Homogenize 2 g of the sample with 5 ml LiCl 20%. Centrifuge. Decant clear layer. Wash residue 2 times with 10 ml portions of water. Filter. Combine clear filtrates and dilute to exactly 50 ml. Pipet 20 ml of this solution into each of two 50 ml volumetric flasks. Add to each flask 5 ml of LiOH-LiCl mixture (respectively 1 M and 0.1 M in strength). Add to one flask 1 ml of a standard formaldehyde solution of 500 ppm. Dilute both flasks to 50 ml. These solutions are ready for the polarographic determination. Purge with nitrogen for 10 minutes into the solution in the polarographic cell. The half-wave potential of formaldehyde is -1.75 Volt. Measure the height of the polarographic wave. Call the height of the diluted sample H1 and the sample with added formaldehyde H2. The formula is:

$$\% \text{ formaldehyde} = \frac{H1 \cdot A}{4W\,(H2 - H1)}$$

, in which A = mg formaldehyde added to the second volumetric flask, and W= g of the sample (in this procedure 2 g).

The standard solutions of formaldehyde can be prepared by diluting 40% formaldehyde solutions with water to approximately 0.05% (500 ppm). The exact strength must then be checked iodometrically according to the European or Dutch Pharmacopoeia. It should be done on the same day as the polarographic determination. The linearity of the polarographic response is good. It was found that linearity exists in the range of 4 to 16 ppm formaldehyde in the final solution.

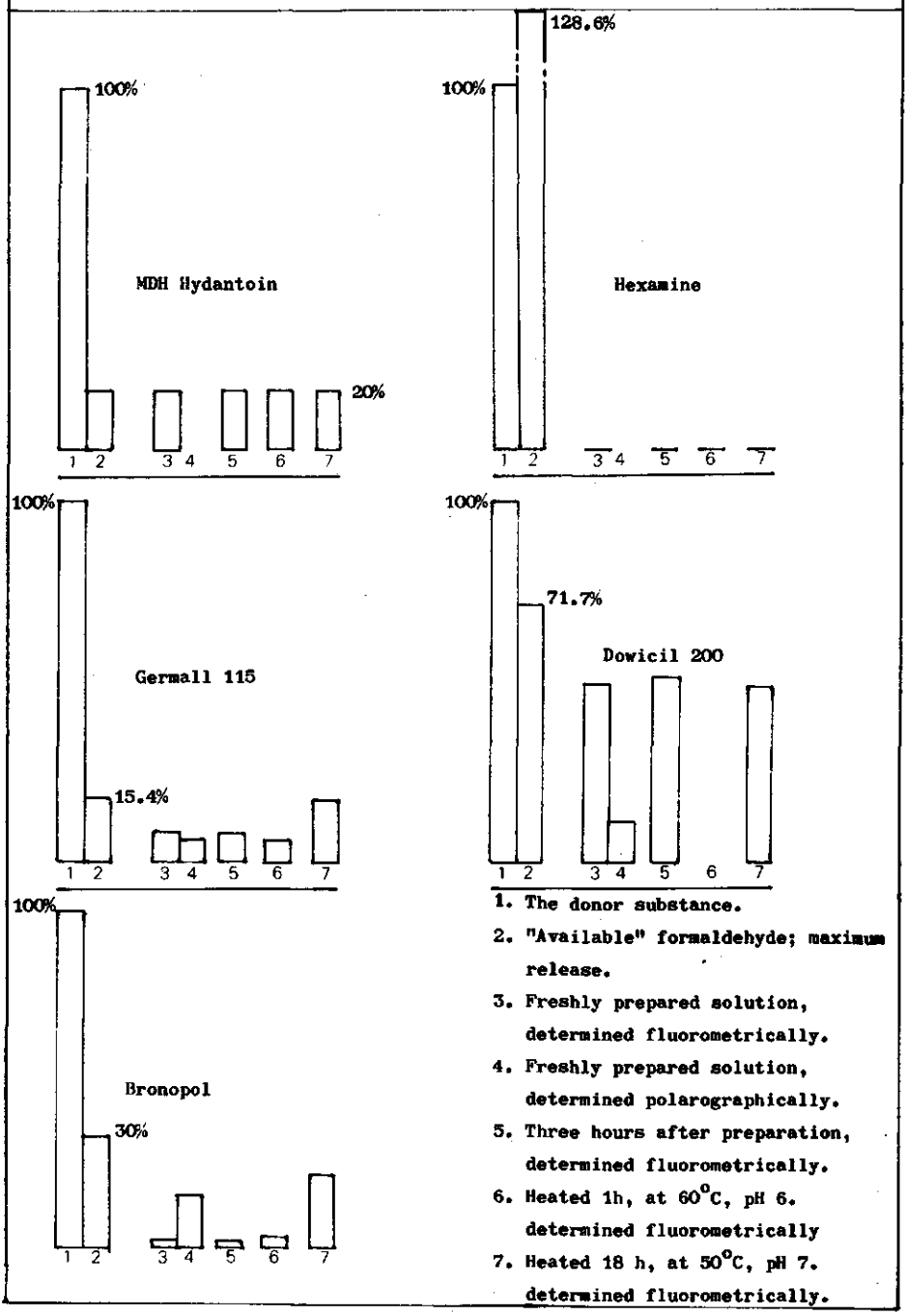
FIGURE 3-W DETERMINATION OF FORMALDEHYDE BY DIFFERENTIAL PULSE POLAROGRAPHY



Formaldehyde donors (group VI) are compounds, which are capable of releasing small amounts of "free" formaldehyde in a formulated product to establish preservative effectiveness, and at the same time reduce the frequency of adverse skin reactions because of the low concentration level of the "free" formaldehyde. Almost no data are available how much "free" formaldehyde is present in a product, that has been preserved by donor compounds. In order to obtain these basic data the following study was made: (a). Maximum release of formaldehyde from the donor compounds Germall 115 and Dowicil 200 was determined by refluxing dilute solutions of each compound and under acid conditions. After cooling the formaldehyde was determined fluorometrically. The data obtained were in good agreement with the theoretical calculations, in which it is assumed that Germall 115 releases 2 mole formaldehyde from its two CH_2OH groups and Dowicil 200 six mole formaldehyde from its hexamine-nucleus (see the chemical formulae of Table 3-XXI).(b) Formaldehyde was determined in dilute solutions of donor compounds immediately after preparation and also after three hours. For the donor compounds Germall 115, Dowicil 200 and Bronopol the determinations were made polarographically. Fluorometric determinations were also made, but for all the five donor compounds. As can be seen on the results in Figure 3-X no significant differences in the data of freshly prepared solutions or in those of the ones determined three hours after preparation can be found. But there are differences for one compound determined by the two methods. The polarographic data (for Germall 115 and Dowicil 200) give a much better picture of the "free formaladehyde" than the fluorometric data, which are much higher. The conclusion can be drawn that during the condensation reaction more formaldehyde was released from the donor compound. Bronopol, however, cannot be compared this way, because of its instability at the high pH of the polarographic determination. (c)The released formaldehyde of several donor solutions after heating at 60°C for one hour with pH 6 has been determined by Sheppard & Wilson (1974). (d) Freshly prepared solutions of donor compounds were also determined by the microdiffusion method, where the solutions were heated for 18 hours at 50°C. The data obtained are also shown in Figure 3-X .

The Figure 3-X as a whole gives a picture of the differences in stability of the five donor compounds. Hexamine and MDH-hydantoin are the two extremes. The first is very stable and will only release formaldehyde at low pH. The liberated formaldehyde at neutral pH is not detectable. As a preservative this donor is not effective and therefore not used much. MDH-hydantoin on the contrary is hardly a donor compound. It immediately releases all the potential amount of formaldehyde. This compound is also hardly used in practice. The other three donor compounds lie between the two extremes. No determinations with Bronidox were made, since this compound was introduced very recently.

FIGURE 3- X STABILITY OF FORMALDEHYDE DONOR-COMPOUNDS.



1. The donor substance.
2. "Available" formaldehyde; maximum release.
3. Freshly prepared solution, determined fluorometrically.
4. Freshly prepared solution, determined polarographically.
5. Three hours after preparation, determined fluorometrically.
6. Heated 1h, at 60°C, pH 6. determined fluorometrically
7. Heated 18 h, at 50°C, pH 7. determined fluorometrically.

Organo-mercurials (group VII) are used in cosmetics at very low concentration levels (0.007 - 0.003 %). Total mercury is determined by flameless atomic absorption techniques (AAS), after mineralizing the organo-mercurial compounds in a closed vessel (Bernas,1968). The procedure for cosmetic products is as follows:
(B) Mineralization of organomercurials in a closed teflon vessel. Destroy 100 mg sample with 3 ml nitric acid 60% by heating for 40 minutes at 150°C. Cool and dilute to 100 ml, and determine total mercury by the flameless AAS method. This procedure was tested with spiked shampoos (phenylmercurynitrate) and yielded 100% for recovery.

Berode et al.(1974)has described the TLC of dithizonates of phenylmercury salts and thimerosal. The isolation from cosmetic products, however, of these dithizonates is not easy. The following isolation procedure for creams or milks was used:
(C) Homogenize a mixture of 1 g sample, 40 ml water, 1 ml NaOH 4M, ca. 2 g sodium chloride. Shake the mixture with 20 ml ether. Discard ether fraction. Acidify with 10 ml sulphuric acid 2.5M. Shake with several 10 ml portions of 0.001% dithizon in CCl_4 until the last extract remains green. Filter collected CCl_4 fractions. Purify by partitioning with 3 x 10 ml 0.025% ammonia and 1 x 10 ml acetic acid 15% succesively. Discard all aqueous fractions. Dry CCl_4 extract by blowing and redissolve residue in 0.5 ml CCl_4. Apply 10 µl per spot on TLC (silica), using benzene-iso-octane (80-15, by vol) as development solvent according to Berode et al.(1974). Orange coloured spots of 0.1 µg are clearly visible. Rf of phenylmercury-dithizonate is 0.50 ; for thimerosal-dithizonate is 0.48; for inorganic-Hg dithizonate 0.40.

Quaternary ammonium compounds (group VIII) are important cosmetic ingredients. Cross (1970) published an excellent review of the analytical techniques for the identification and determination of cationic surfactants, to which the quats belong. Detection of a quat in a cosmetic can be most conveniently done by the formation of a pink permanganate complex which is soluble in chloroform. Pyridinium and isoquinolinium compounds will form unstable complexes with permanganate, which fade after 30 seconds. But those of the other quats are stable for several minutes. The following procedure is suitable for the detection of a quat in a cosmetic:
(D) Shake 2 ml of the diluted sample, containing approximately 0.05 % quat, with a little acetic acid,(the pH must then be approximately 3) and 5 ml 0.25% $KMnO_4$ solution and 2 ml chloroform. Shake vigorously for 5 seconds. If a quat is present a pink brown colour will appear in the chloroform layer as soon as the phases separate.

Several analytical techniques can be used to identify the quat compound. It can be done by two-dimensional TLC, such as has been described by Gabriel (1974). Gas-chromatography of quats is based on prior-fragmentation into compounds of greater volatility (Warrington, 1961) or on "on-column degradation" by alkaline columns (Metcalfe, 1963).

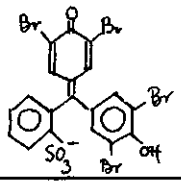
Quantitative determination can be done by titration. The large cat-ion of the quat reacts stoichiometrically with a large anion, which can be a reineckate,

FIGURE 3- Y

ANALYSIS OF QUATERNARY AMMONIUM COMPOUNDS

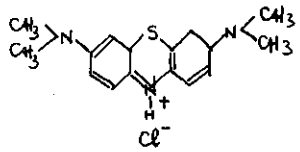
A Formula of an anionic dye-indicator

BROMTHYMOLBLUE



B Formula of an cationic dye-indicator

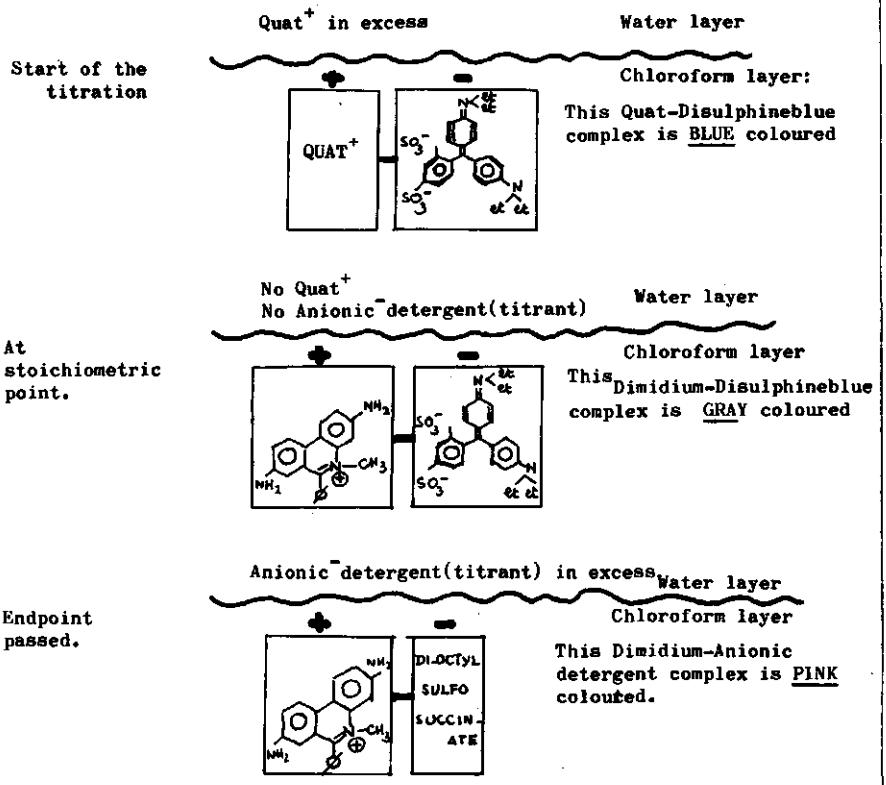
METHYLENEBLUE



C Schematic diagram of a two-phase titration system with mixed-indicator

Dimidium bromide - Disulphine blue VM

$(cationic)^+$ $(anionic)^-$



hexanoferrate-III, phosphotungstate, tetraphenylboron or an anionic dye (e.g. methylorange, bromophenolblue), or an anionic detergent. If the cation-anion complex is insoluble in water, such a precipitation can be weighed (gravimetric analysis). If an excess of the anionic detergent titrant has been added, this excess can be determined volumetrically after removal of the precipitate. If the anion is a dye, the quat-dye complex can be dissolved in an organic solvent and measured colorimetrically (Chatten & Okamura, 1973). If an anion-titrant, such as tetraphenylboron (kalignost) is added gradually from a burette to the quat, the stoichiometric point is visually detectable by the sudden change in colour of an indicator dye (such as dichlorofluoresceine). The colour change is caused by the disappearence of the quat as a solubilizing agent for the water-insoluble dye indicator. An example of this "one-phase" titration of quats is described by Metcalfe et al.(1966). More practiced - in contrast to "one-phase" titrations - are the "two-phase" titration methods. An anionic-detergent titrant is added from the burette to the quat, and chloroform as a second phase is introduced during titration. A dye-indicator, either an anionic or a cationic type, is necessary to detect the endpoint. If the dye-indicator is anionic (methylorange, bromthymolblue) the colour complex of a quat-anionic dye remains in the chloroform phase, which is coloured at the beginning of titration. At the end of titration this colour disappears from the chloroform. If the dye-indicator is cationic (methyleneblue) the chloroform layer is colourless at the beginning of titration. It will however be coloured at the endpoint. due to the availability of the excess anionic detergent titrant, which couples with the cationic dye indicator to form a coloured complex which is soluble in chloroform. These "two-phase" titrations were introduced by Hartley and Runnicles (1938), and are commonly known as Epton or Barr titrations. A lot of work has been done to improve the sharpness of the endpoint of these "two-phase" titrations. A collaborative study was made in 1967 by the Commité International de Dérivé Tensio-actives, to review and compare the many modifications introduced since the work of Hartley and Runnicles. They came to the conclusion that a mixed indicator as published by Herring (1962) gives the sharpest endpoint. The mixed indicator system introduced by Herring is a mixture of cationic dye (dimidiumbromide; see Figure 3-Y)and an anionic dye (disulphineblue VM). At the start of the titration the chloroform layer is blue with the quat-disulphineblue complex. At the stoichiometric endpoint neither the quat nor the anionic titrant is available. At that point the chloroform layer is only grey by the dye-indicators complex (dimidium-disulphineblue). The next drop of the anionic detergent titrant will cause a pink colour of the chloroform which is clearly visible and which is the colour of the dimidium-anionic detergent complex. A schematic diagram of this mixed-indicator system is given in Figure 3-Y.

Dimethoxane (GivGard DXN CO) is the first compound of the rest-group (group IX). The compound is an acetaldehyde donor, and can be detected by TLC on silicaplates, using the solvent mixture ethylacetate-methanol-ammonia (65-30-5, by vol.). After spraying with a solution of 2.4.dinitrophenylhydrazine reagent the reference substance give two spots with Rf 0.7 and 0.9. Dimethoxane is however not detectable after a short time when used as a preservative in cosmetic creams and milks.

Chlorhexidine (Hibitane) can be determined by methods described by Cropper et al.(1975)and by Siefert et al.(1975). In one of the methods chlorohexidine is hydrolized to p.chloraniline and at the same time diazotized, followed by conversion to 1.4.-iodochlorobenzene, which can be extracted into n.hexane and finally analysed by electron-capture gaschromatography.

Pyridinethion (Omadine) is an anti-dandruff agent and used as a soluble sodium or an insoluble zinc salt in shampoos or hair dressings. These salts can be extracted by hydrochloric acid or by EDTA from the products which is then precipitated as the copper or ferric chelate, then dissolved in chloroform and finally determined colorimetrically. (Kabacoff & Fairchild,1975).

EDTA (ethylenediaminetetraacetic acid) was determined by the method of Cherney et al.(1954), which was adapted for the analysis of aqueous cosmetics and contact lens fluids.

3.7.3. Analytical results of the "antimicrobials" market survey.

A market survey of antimicrobials in many kinds of cosmetic products was made in 1974. The results are tabulated in Table 3-XXIV. It can be seen on that table that the paraben esters and formaldehyde (the donors included) are the most important preservatives used in cosmetics. The halogenated phenols are frequently used for their antiseptic (deodorizing and anti-dandruff included) properties, in particular hexachlorophene, dichlorophene and triclosan. In the market survey contact lense fluids were also included. Preservation of these products is mainly done with organo-mercurials, quaternary ammonium compounds and EDTA.

TABLE 3-XXIII ANALYTICAL RESULTS OF THE "ANTIMICROBIALS" MARKET SURVEY. 1974-1975.

		* .1	.2	.3	.4	.5	.6	.7	.8	.9	.10
	Number of samples	(12)	(87)	(11)	(20)	(11)	(29)	(32)	(6)	(11)	(19)
I	Parabens	.11	.71	.	.	.	.5	.	.	.	.
	Thymol	.	.	.	.	.	.3	.	.	.	.
	BHA	.	.4	.	.	.	.	.	.	.	.
	unidentified phenols	.	.2	.	.	.1	.2	.4	.	.3	.
II	Hexachlorophene	.	.4	.	.	.4	.10	.12	.3	.4	.
	Dichlorophene	.	.3	.	.	.	.10	.	.	.	.
	Triclosan	.	.	.	.	.	.3	.10	.1	.1	.
	PCMX	.	.1	.	.	.	.	.	.	.	.
III	TCC	.	.	.	.	.	.1	.	.	.2	.
	Cloflucarban	.	.2	.	.	.1	.	.2	.	.	.
	Trichlorbutanol	.	.	.	.	.	.	.	.	.	.1
	Chloracetamide	.1	.1	.	.	.	.	.	.	.	.
IV	Sorbic acid	.1	.	.	.	.	.	.	.	.	.
	Salicylic acid	.	.	.	.	.	.2	.	.	.	.
	Undecylenic acid	.	.	.	.	.	.9	.	.	.	.
V-VI	Formaldehyde("free") (incl.donors)	.5	.30	.7	.13	.2	.3	.	.	.	.
VII	Thimerosal	.	.	.	.	.	.	.	.	.	.9
VIII	Quat	.	.	.	.	.	.	.	.	.	.9
IX	Chlorhexidine	.	.	.	.	.1	.6	.	.2	.	.
	Dimethoxane	.	.2	.	.	.	.	.	.	.	.
	EDTA	.1	.	.1	.3	.	.	.	.	.	.14
	Phenoxetol	.1	.	.	.	.	.3	.	.	.	.
	Pyridinethion	.	.	.	.	.4	.	.	.	.	.

*
Products: 1 = Skin creams and milks.
2 = Eye make-up.
3 = Bubble baths.
4 = Shampoos.
5 = Anti-dandruff shampoos
6 = Foot products.
7 = Deodorant sprays.
8 = Intimate sprays.
9 = Soaps.
10 = Contact lense fluids.

4. Conclusions.

It is hoped that the results of this study of the analysis of several classes of risk-bearing compounds may have made two important contributions towards the improvement of the general safety of cosmetics.

In the first place analytical methods have been developed for some of the actually occurring risk-bearing compounds in marketed cosmetics. The analytical experience gained in this study can be used in the development of official methods. In the light of recent tendencies in current legislations the need for analytical control of negative, positive lists becomes more urgent. In fact parts of this study have recently been presented as contributions to the EEC Working Group for the Analysis of Cosmetics.

The second contribution of the data obtained from the market surveys is the identification of actually used members in the different classes of risk-bearing compounds. Such data are very important for toxicologists in determining the priorities in the "gap filling" toxicological research. Toxicological work is very expensive and therefore priorities should be carefully determined, in order to establish the shortest and most efficient way to a cosmetics GRAS ("generally recognized as safe") list of ingredients.

One must, however, realize that the knowledge of actually used members of the different classes of risk-bearing compounds can also be obtained by voluntary or mandatory regulations of the registration of products composition by manufacturers. For instance the US Voluntary Cosmetics Regulatory Programme, which was started in started in 1974, may be able to compile such data. The same situation can be expected in the EEC-member states, in the near future, if formula disclosure becomes mandatory.

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