Vol. 26 No. 5 May 1975

Journal of the Society of Cosmetic Chemists

Contents

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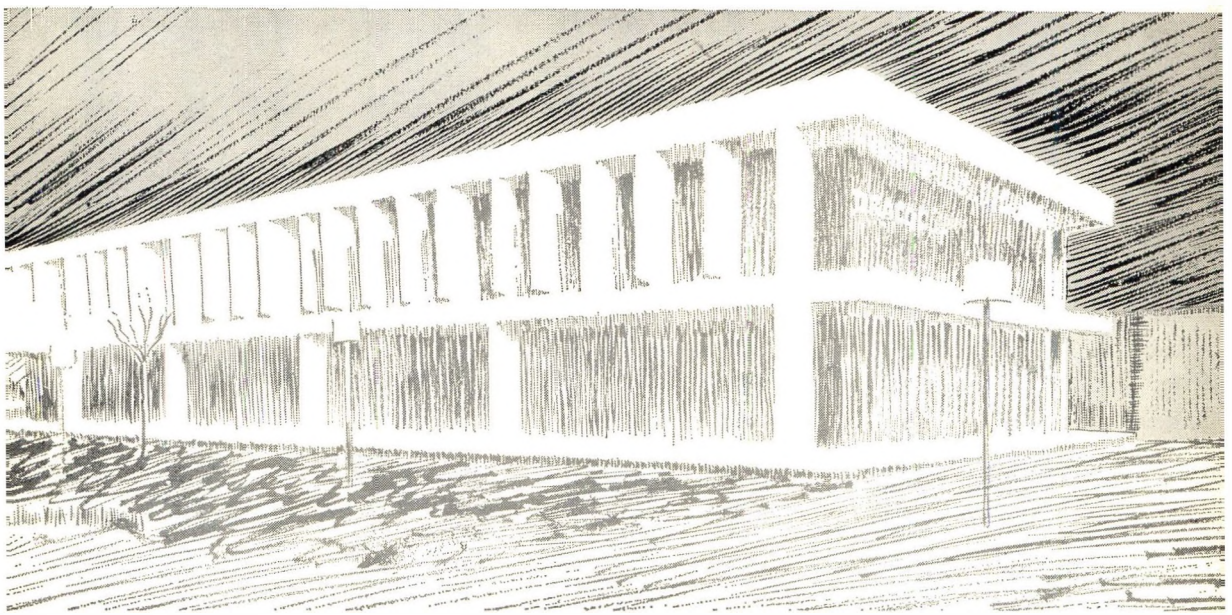

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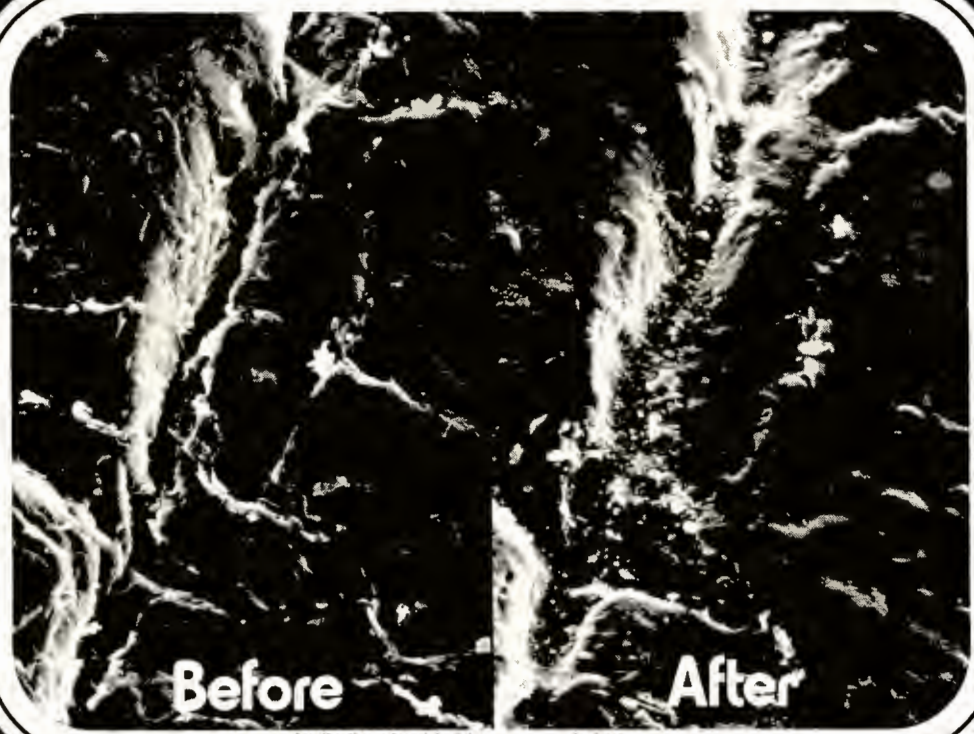

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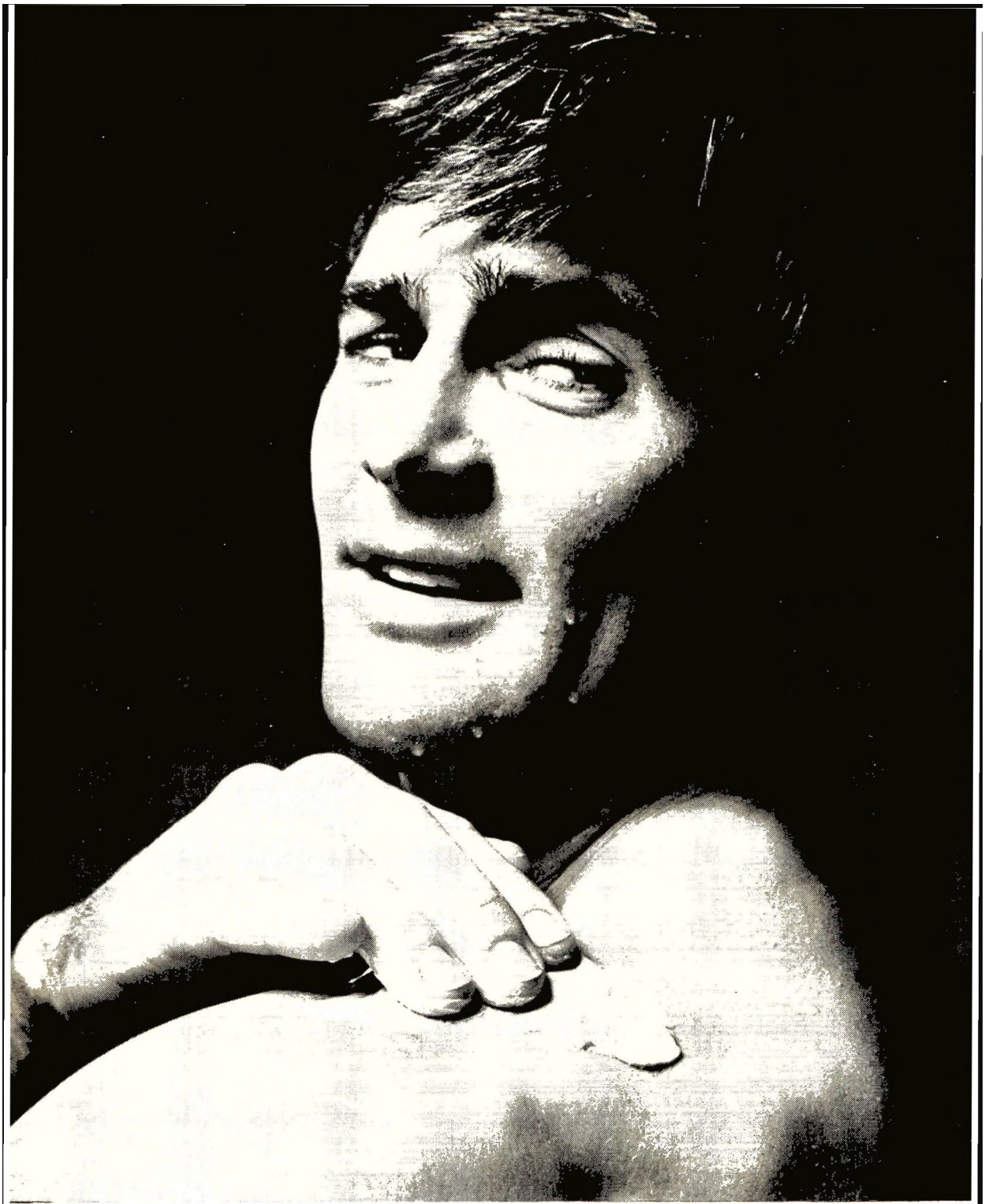

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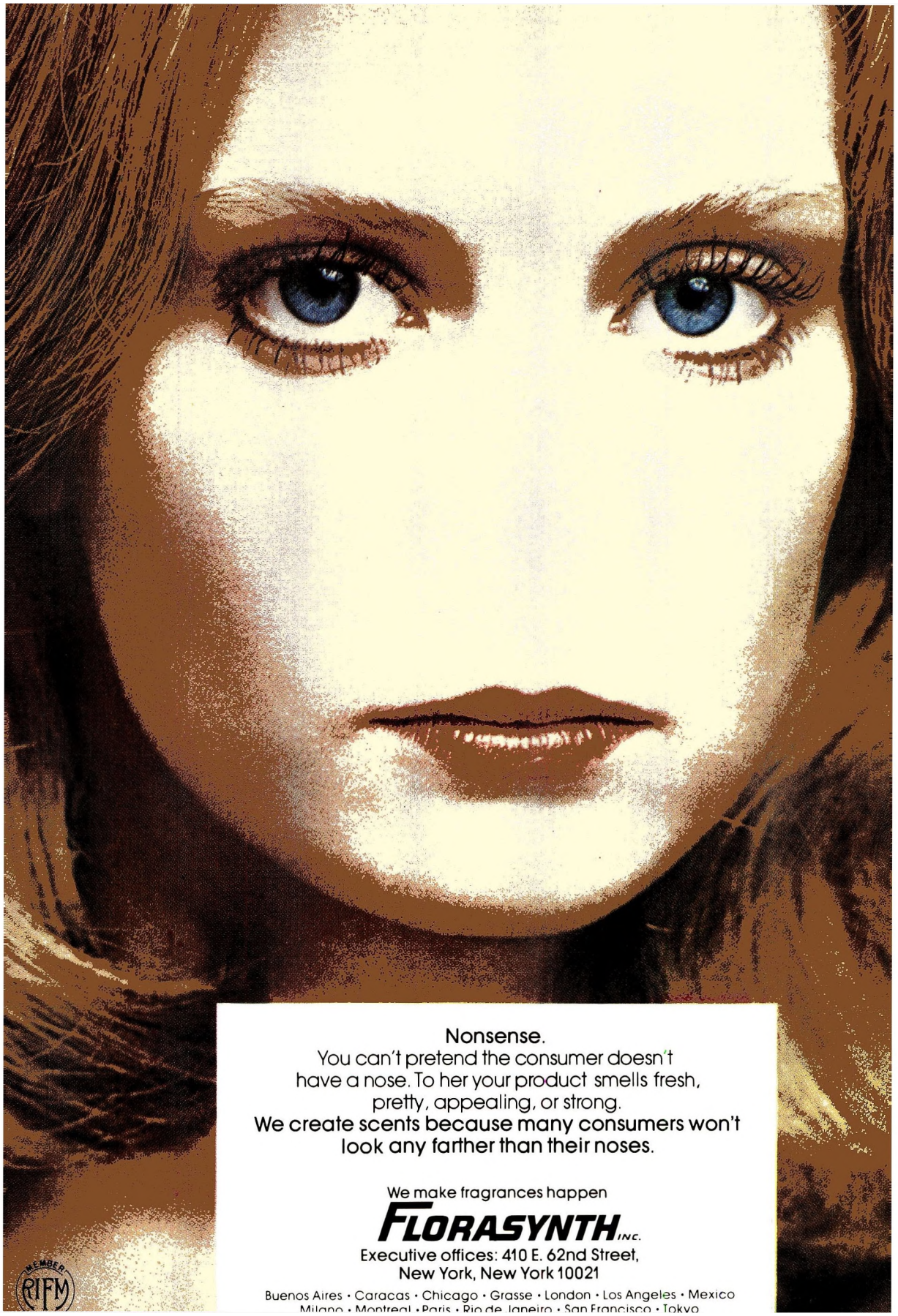
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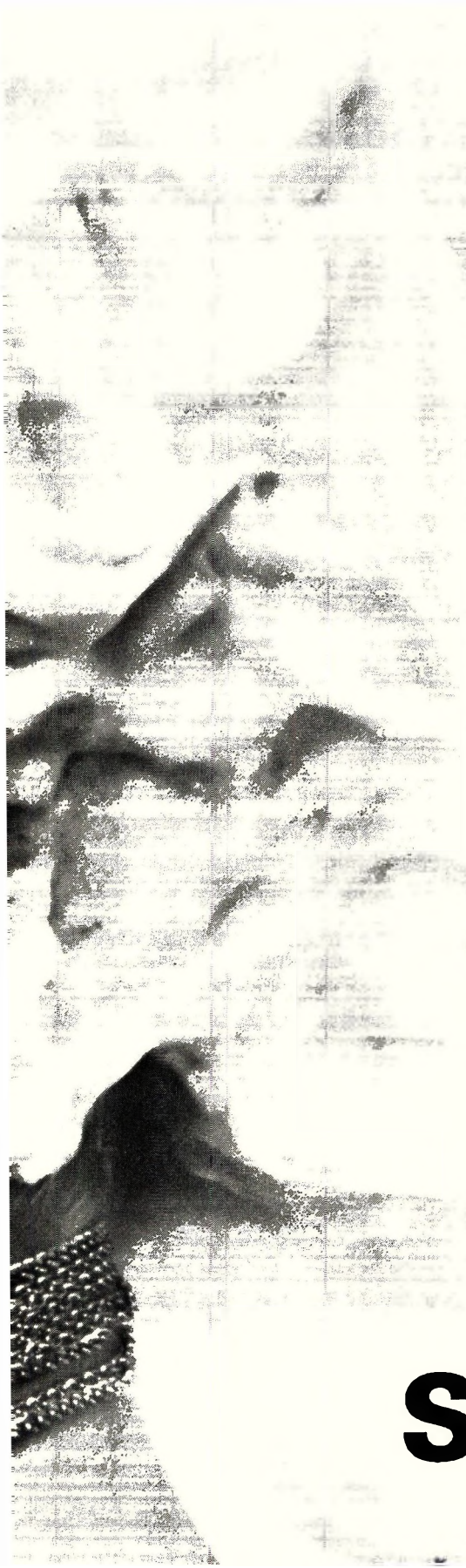

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SYNOPSES FOR CARD INDEXES

The following synopses can he cut out and mounted on 3 x 5 in. index cards for reference, without mutilating the pages of the Journal.

Clinical evaluation of baby oil as a dermal moisturizer: Eugene R. Jolly and Charles A. Sloughfy. *Journal of the Society of Cosmetic Chemists* **26,** 227 (May 1975)

Synopsis—A population of 106 adult females who exhibited roughened, cracked, and inflamed skins of the elbows, knees, shins, and heels were selected for the evaluation of the skin moisturizing actions of baby oil. The baby oil was applied at least twice daily during a 4-week study period to the specified sites on one side of the body. The contralateral sites served as untreated controls. Ratings of the skin sites at the outset of the study, during a 2-week interim examination, and at the end of 4 weeks defined a marked and progressive improvement of sites treated with the baby oil. Conversely, untreated sites tended to worsen during the study period.

A statistical analysis of the data revealed that the moisturizing actions were highly significant at both the 2- and 4-week examination times. All ratings were made without foreknowledge of which side of the body had been treated. Photographs obtained in 10 subjects clearly illustrated the differences between skin of treated versus nontreated sites. Following discontinuance of the baby oil application, skins of the treated sites tended to become drier within the first 48 hours. These data suggest that the skin moisturizing actions of the baby oil are not protracted.

So-called odor eliminators: Ernst Paukner and V. Hudewenz. *Journal of the Society of Cosmetic Chemists* **26,** 235 (May 1975)

Synopsis—The elimination of unpleasant odors by cosmetics or in the home or in technical products is a continuing problem. Commercially available odor eliminators exhibit an effect which presumably depends on chemical reactions. It is also frequently claimed that odor eliminators act selectively on unpleasant smelling materials. Therefore, the degree to which malodorous or pleasant smelling materials are eliminated from the gas phase by use of odor eliminators and the mechanism by which this removal occurs were studied. The extremely complicated infared procedures for determining chemical bonding gave indication of molecular, ionic, or hydrogen bonding. Experiments in which the interaction of the odor eliminator with a gaseous odorant is carried out by aerosolization simulate the practical application of a room spray. The decrease of odorant concentration in the gas phase after application of an odor eliminator and specific solvents was quantitatively determined by gas chromatography. It was shown that the so-called odor eliminators have no selectivity for malodorous materials in this case.

Instead, the decrease of odor depends on physical dissolution, i.e., a washing effect. The same effects can be achieved with certain solvents, and a modestly greater decrease in odorant concentration can occur in the presence of film-formers. The latter may envelop suspended odorant droplets and attach odoriferous molecules to a precipitate.

Isolation and identification of the protein component of hair melanin: Kathleen Hall and Leszek J. Wolfram. *Journal of the Society of Cosmetic Chemists* **26,** 247 (May 1975)

Synopsis—Electron microscopic studies have previously revealed that, in the melanocyte, granules appear to exist in varying stages of development. They represent preliminary synthesis of nonmelanized granules and are composed of coiled protein filaments or membranes. This protein matrix serves as a substrate for subsequent deposition of the melanin polymer and thus becomes totally encapsulated by the pigment. Upon reaching complete melanization, the granules appear uniformly dense, and no internal structure can be resolved by conventional techniques.

Previous attempts to identify the melano-protein have been hindered by the chemical inertness of the melanin capsule. A technique for solubilizing the melanin was found and provides a facile method of isolating the protein moiety present in the pigment granule. The chemical composition of this protein has also been determined.

A critical comparison of two procedures for antiperspirant evaluation: W. M. Wooding and Paul Finkelstein. *Journal of the Society of Cosmetic Chemists* **26,** 255 (May 1975).

Synopsis—This paper is concerned with experimental design and data analysis procedures associated with gravimetric axillar antiperspirant tests. The most widely known of these tests requires the estimation of "control ratios" for each subject, based upon data obtained prior to the evaluation of product application results. These ratios are subsequently employed as correction factors to modify similar ratios in sweating responses, which are calculated during the product evaluation phase of the test. It will be shown that an improved procedure, which has been in use by this laboratory for more than 7 years, is substantially more economical with regard to time and effort than the above procedure. The new procedure does not require preliminary testing or the establishment of "control ratios". In addition, it is conceptually more rigorous, allows more definitive conclusions to be drawn, and conforms to established principles of statistical design and analysis.

In summary, it is claimed that the new method is easier, faster, more economical, and can be shown to be unbiased. The use of these two procedures will be illustrated with examples.

J. Soc. Cosmet. Chem., 26, 227–234 (May 1975)

Clinical Evaluation of Baby Oil as a Dermal Moisturizer

EUGENE R. JOLLY, M.D., Ph.D. and CHARLES A. SLOUGHFY*

Synopsis—A population of 106 adult females who exhibited roughened, cracked, and inflamed skins of the elbows, knees, shins, and heels were selected for the evaluation of the SKIN MOISTURIZING ACTIONS of BABY OIL. The baby oil was applied at least twice daily during a 4-week study period to the specified sites on one side of the body. The contralateral sites served as untreated controls. Rating of the skin sites at the outset of the study, during a 2-week interim examination, and at the end of 4 weeks defined a marked and progressive improvement of sites treated with the baby oil. Conversely, untreated sites tended to worsen during the study period.

A statistical analysis of the data revealed that the moisturizing actions were highly significant at both the 2- and 4-week examination times. All ratings were made without foreknowledge of which side of the body had been treated. Photographs obtained in 10 subjects clearly illustrated the differences between skin of treated versus nontreated sites. Following discontinuation of the baby oil application, skins of the treated sites tended to become drier within the first 48 hours. These data suggest that the skin moisturizing actions of the baby oil are not protracted.

Introduction

According to Blank (1), primary therapy for prophylaxis or correction of dry, scaly, eroded, and enflamed skin should be directed toward maintenance of optimum hydration of stratum cornea. Studies have shown (2, 3) that natural skin lipids are not very effective in preventing water loss under conditions of low environmental humidity. Nor can passage of water through the stratum lucidum and stratum granulosum, the preservers of body water and electrolytes, compensate for enhanced evaporation.

Jellinek (4) points out that removal of surface lipids through frequent use of detergents or exposure to organic solvents also facilitates skin drying. He stresses that moisture in the stratum corneum is bound by hydrophilic components including amino acids, pentoses, and phospholipids. If the skin is

*Biometric Testing, Inc., Englewood Cliffs, N.J.

stripped of lipids, then these hydrophilic modules are readily washed out. In addition, when the fat layer is removed, the surface of the stratum corneum becomes roughened, exposing greater areas of skin, and speeding the evaporation process.

Powers and Fox (5) report that emollient products, formulated to maintain skin integrity under conditions of excessive water loss, vary markedly in effectiveness. Utilizing silica gel dessicators, these investigators studied a series of materials for effectiveness in retarding skin dehydration. Many of the formulations, labeled as skin softeners, actually increased water loss, one as much as 56%. Petrolatum United States Pharmacopoeia (USP) was the most effective barrier material tested for maintenance of dermal hydration.

Anhydrous lanolin, mineral oil, and cold cream also proved to be effective barriers. Spruitt (6) also quantitated water loss from the skin following application of a variety of materials. Flow of water was decreased from 500- to 1000-fold by aliphatic products (mineral-oil based). Unsaturated vegetable oils provided only slight protection, whereas saturated fats were intermediate in activity. Emulsions tended to increase evaporation. Eisner's (7) results support those of Fox and Powers and of Spruitt. Ointments or lotions containing humectants and surfactants again proved ineffective barriers; in this case, with regard to the penetration of an experimental film model by a series of noxious materials including urine, fecal suspensions, acids, and bases. Conversely, baby oil and petrolatum prevented passage of the noxiants.

Baby oil with a mineral oil base remains perhaps the most widely employed skin emollient or softener. In spite of its hydrophobic properties, assuming that the principles of therapy expressed by Blank and other investigators are valid, baby oil can also be properly termed a skin moisturizer. Published data suggest that baby oil as a liquid hydrocarbon, that is capable of providing an effective barrier against water loss, would provide superior moisturizing actions, and that its utility in protecting the vulnerable skin of infants would be equally apparent after application to adults who exhibit sequelae to the variety of skin trauma induced by mechanical and environmental assaults.

The present study was designed to clinically define the moisturizing actions of a commercially-available baby oil* when applied to the roughened, cracked, and inflamed skins of the elbows, knees, heels, and shins of adults.

Experimental Methods

The study was conducted using a single-blind design with the subjects serving as their own controls. The study group was composed of 111 females with an age range of 13 to 72 years and an average age of approximately 31 years. Treatment sides and contralateral control areas (elbows, knees, heels,

*Supplied by Baby Products Company, Johnson & Johnson, New Brunswick, N.J.

and shins) were randomized according to a schedule developed prior to initiation of the study. To be eligible for participation, candidates were required to be free of any dermatological pathology related to systemic diseases and to exhibit severity of symptoms conforming to a rating of 2 or higher on a 5-point rating scale for at least 2 of the 4 specified skin sites as follows.

A. Elbows and knees
0=smooth visually and to the touch
1="snow" visible in creases; slightly rough to the touch
2=scales and "snow" visible; moderately rough to the touch
3=pronounced scaling and roughness to the touch
4=cracking associated with severe roughness to the touch

B. Heels
0=smooth visually and to the touch
1=rough to the touch with "snow" and cracking just visible
2=rough to the touch; cracking and scaling evident
3=quite rough to the touch; scaling and cracking pronounced
4=very rough to the touch; extreme scaling and cracking

C. Tibia
0=smooth visually and to the touch
1=smooth to the touch; "snow" visible in creases
2=scaling and slight glazing
3=pronounced scaling and slightly rough to the touch
4=severe scaling and cracking; very rough to the touch

Following the baseline examination, subjects were instructed to utilize at least 2 drops of the baby oil on the designated areas at least twice daily (on arising and at bedtime) during a 4-week study period. While the oil was to be applied sparingly so that it was not recognizable at the times of clinical assessment, they were requested to use enough to cover the test sites completely. Use of other skin conditioning preparations during the study was prohibited. The subjects were again rated after 2 and 4 weeks. All examinations were conducted in a uniform manner with elbows and knees bent at a 90° angle. Discussions between the investigator and subject were prohibited, and at no time was the investigator made aware of the identity of the treatment side.

Finally, photographic documentation of effectiveness was obtained from 20 of the subjects. These individuals had been selected at random and specified by subject number prior to therapeutic trial. Each of the 4 sites on both the right and left sides of the subjects were photographed at the scheduled examinations and again either 24 or 48 hours following treatment withdrawal to allow a determination of duration of baby oil activity.

Results

Valid data were obtained from 106 of the initial 111 participants. Four subjects were excluded from the final statistical analysis because of insufficient baseline dermatologic involvement and one because of insufficient rating data due to a missed rating session caused by intercurrent illness.

That the initial severity of the dermatological signs were comparable for the treatment sites and contralateral untreated sites among the population can be ascertained by review of Table I. In general, skin of elbows, knees, and heels exhibited a greater degree of drying, scaling, and roughness at the outset of the trials in comparison to shins.

Skin moisturizing activities of the baby oil are illustrated in Fig. 1. Severity of the signs was decreased in comparison to baseline readings to a significant extent at all 4 treatment sites. Conversely, untreated sites tended to become more severely afflicted over the 4-week study period. Actually, tibial areas were significantly worse at the 4-week evaluation (P=0.01). Improvement appeared to be progressive with continued treatment. However, even after 2 weeks, the degrees of benefit were clearly demonstrable. A summary of the statistical analyses of the data is presented on Table II.

Results of evaluations of the 10 individuals who returned to the clinic 24 hours after the baby oil applications were terminated and the additional 10 who reported to the clinic 48 hours later are summarized in Table III.

The data suggest that the protectant moisturizing actions of baby oil do not persist for extended periods following termination of therapy, as skin of treated sites tended to return to the pretreatment state during the 24- and 48-hour follow-up periods.

Mean scores calculated from raw data and obtained utilizing the described objective methods prove the thesis that the completely nonpolar liquid hydro-

Table I

Distribution of Sites by Treatment Group and Initial Severity of Dermatologic Signs

	Site							
	Elbow		Knee		Heel		Tibial Area	
Severity[a]	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated
0	5	3	7	9	4	4	6	7
1	24	27	40	35	20	20	48[b]	49[b]
2	53[b]	52[b]	48[b]	50[b]	62[b]	59[b]	43	41
3	20	20	10	11	18	21	9	9
4	4	4	1	1	2	2	0	0
TOTALS:	106	106	106	106	106	106	106	106

[a] Median severity.

[b] See protocol for definitions.

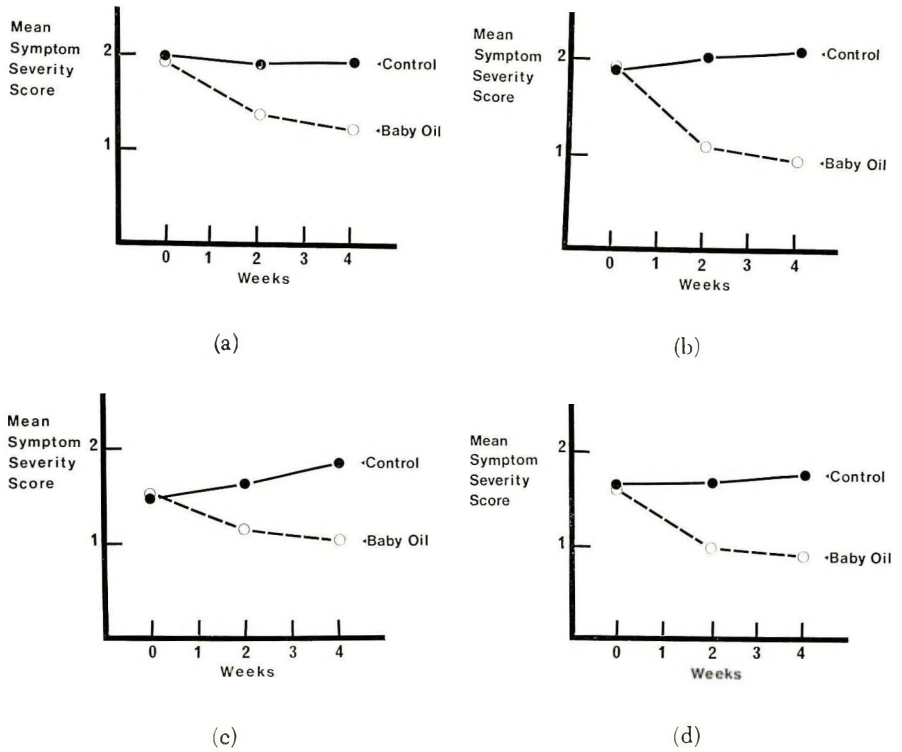


Figure 1. Reduction in mean symptom scores following application of baby oil to various anatomical sites: (a) heels; (b) elbows; (c) shins; (d) knees

Table II
Mean Dermatologic Severity
(Scores By Weeks)

	Baseline Score	Week 2 Mean	Week 2 Statistical Significance[a]	Week 4 Mean	Week 4 Statistical Significance[a]
Elbows					
treated	1.9	1.1	0.001	1.0	0.001
untreated	2.0	2.0	0.311	2.1	0.066
Knees					
treated	1.6	1.0	0.001	0.9	0.001
untreated	1.6	1.7	0.543	1.7	0.245
Heels					
treated	1.9	1.4	0.001	1.2	0.001
untreated	2.0	1.9	0.538	1.9	0.588
Tibial Areas					
treated	1.5	1.2	0.001	1.1	0.001
untreated	1.5	1.6	0.213	1.7	0.001

[a] Statistical significance of change from baseline—Wilcoxon Matched-Pair Signed Ranks.

Table III

Mean Severity Ratings 24 and 48 Hours After Treatment as Compared to the Ratings for the Last Treatment Day[a]

	Mean Severity of Treated Sites				Mean Severity of Untreated Sites			
	Last day of treatment	24 hours after	Last day of treatment	48 hours after	Last day of treatment	24 hours after	Last day of treatment	48 hours after
Elbows	1.4	2.1	0.9	1.8	2.6	2.6	2.3	2.2
Knees	0.7	1.8	0.7	0.9	2.6	2.5	1.2	1.4
Heels	1.2	1.8	1.3	1.9	2.7	2.8	2.2	2.4
Shins	1.0	1.9	0.9	1.6	2.4	2.4	1.7	1.7

[a] Two separate treatment groups composed of 10 subjects each were evaluated at 24 and 48 hours, respectively.

carbon, as represented by the baby oil, can exert clearly demonstrable skin moisturizing action. This anomaly was even more impressively demonstrated photographically. Figures 2 and 3 illustrate contrasts between treated and untreated sites.

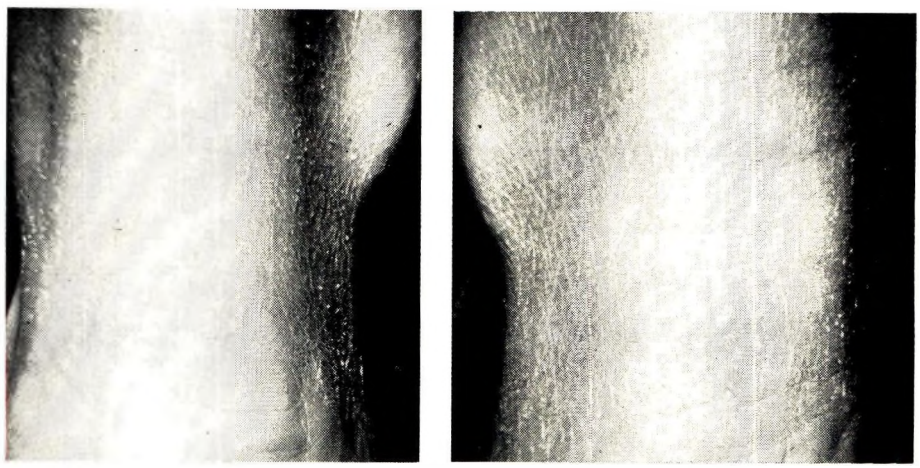
Review of photographs from all 20 subjects allowed definition of similar degrees of benefit. Conversely, the follow-up photographs taken at 24 and 48 hours later exhibited less remarkable differences. The photographs, like the ratings, indicate that deterioration is relatively rapid when applications of the baby oil are stopped.

Discussion

An occlusive film of a liquid hydrocarbon as afforded by the baby oil, according to available evidence, provides a highly effective mechanism for retarding water loss even under extremes of low humidity in the environment. Such a product, then, can appropriately be termed a skin moisturizer despite its hydrophobic properties. In fact, occluding the skin may be a more effective mechanism for maintaining moisture and protecting the stratum corneum than attempts to promote penetration and skin retention of hydrophilic substances (e.g., amino acids and polypeptides) and/or oleaceous materials.

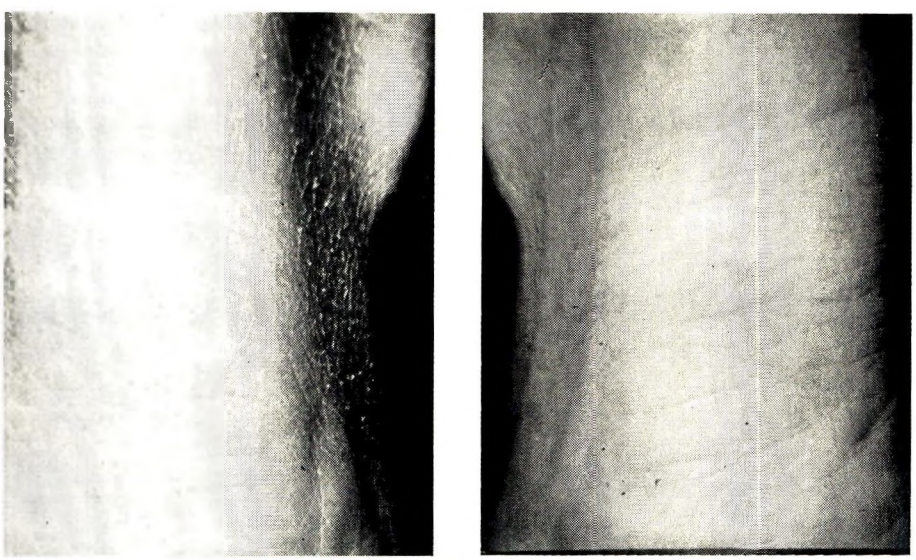
The data obtained in the present study are clearly the most impressive that we have obtained in our clinic, although we have had the opportunity to evaluate many "moisturizers," of which several were apparently effective.

(Received October 10, 1974)



(a) (b)

Figure 2. Comparison of (a) right and (b) left heels prior to treatment



(a) (b)

Figure 3. Comparison of (a) right and (b) left heels following 4 weeks of baby oil treatment of left side

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Die sogenannten Geruchsvernichter II *

ERNST PAUKNER ** und V. HUDEWENZ **

Nach einem Vortrag anläßlich des 8th Congress of the International Federation of Societies of Cosmetic Chemists (IFSCC) am 27. August 1974 in London

Synopsis — So-called odor eliminators. — The ELIMINATION OF UNPLEASANT ODORS by COSMETICS or in the home or in TECHNICAL PRODUCTS is a continuing problem. Commercially available odor eliminators exhibit an effect which presumably depends on chemical reactions. It is also frequently claimed that odor eliminators act selectively on unpleasant smelling materials. Therefore, the degree to which malodorous or pleasant smelling materials are eliminated from the gas phase by use of odor eliminators and the mechanism by which this removal occurs were studied. The extremely complicated INFRARED PROCEDURES FOR DETERMINING CHEMICAL BONDING gave NO INDICATION OF MOLECULAR, IONIC, OR HYDROGEN BONDING. Experiments in which the interaction of the odor eliminator with a gaseous odorant is carried out by aerosolization simulate the practical application of a room spray. The decrease of odorant concentration in the gas phase after application of an odor eliminator and specific solvents was quantitatively determined by gas chromatography. It was shown that the so-called odor eliminators have no SELECTIVITY for malodorous materials in this case. Instead, the decrease of odor depends on PHYSICAL DISSOLUTION, i.e., a WASHING EFFECT. The same effects can be achieved with certain solvents, and a modestly greater decrease in odorant concentration can occur in the presence of FILM-FORMERS. The latter may ENVELOP SUSPENDED ODORANT DROPLETS and ATTACH ODORIFEROUS MOLECULES TO A PRECIPITATE.

Man ist sich bisher nicht darüber im klaren, wie der Mechanismus der immer wieder behaupteten und subjektiv festgestellten Geruchsvernichtung beschaffen ist. Oft wird angegeben, Geruchsvernichter (abgekürzt: GV) wirkten selektiv auf schlechte Gerüche. Der Grund seien entweder chemische Umsetzungen oder bestimmte Bindungskräfte. Lowicki (1) hat hierzu Theorien ausführlich dargestellt. Es sei schon jetzt vorweggenommen, daß weder eine Molekülbindung

* I. Mitteilung vgl. Zitat (2).

** drom, D-8021 Baierbrunn im Isartal.

durch chemische Reaktion oder eine Ionen- bzw. Wasserstoffbrückenbindung, noch eine Bindung durch van der Waal'sche Kräfte im Spiel sind. Der Sachverhalt ist ausnahmsweise viel einfacher.

1. AUFGABENSTELLUNG

In einer früheren Arbeit (2) hatten wir durch objektivierbare Messungen mit einem Präzisionsolfaktometer gefunden, daß sich die Wahrnehmbarkeit einer Riechstoff/GV-Mischung gegenüber dem Riechstoff allein etwas verringert, wenn beide Komponenten Zeit zur Einwirkung aufeinander hatten. Die seinerzeit noch nicht möglich gewesene Erklärung des Phänomens kann jetzt gegeben werden. Gegenstand der Untersuchung ist die Prüfung, ob Stink- und Riechstoffe bei Anwendung von GV und anderen Substanzen aus der Gasphase eliminiert werden können und welcher Mechanismus im zutreffenden Falle der Eliminierung zugrunde liegt.

2. VERSUCHSANORDNUNGEN

2.1. IR-Spektroskopie

Da die bisher — meist nur in Produkt-Prospekten (3) — aufgestellten Theorien der Geruchsvernichtung experimentell nicht bewiesen sind, wurde zunächst versucht, die immer wieder behauptete chemische Bindung infrarot-spektroskopisch nachzuweisen. Dies ist experimentell sehr schwierig, weil GV in ihren Lösungsmitteln nicht untersucht werden können, denn das Spektrum des Lösungsmittels (meist Äthanol) überlagert das Spektrum der übrigen Substanzen. Auch der geringe Dampfdruck der GV gehört zu den Ursachen fehlender Beweiskraft dieser spektralen Informationen.

Bei einem GV wie Produkt C***, das frei von Lösungsmitteln ist, konnte bei Einwirkung auf Diäthylsulfid*** keine Veränderung am Spektrum erkannt werden. Auch die Vermessung des auf das Natriumchlorid-Fenster gesprühten Films von Diäthylsulfid und C führte zu keinem Hinweis etwa auf Ionen-, Molekül- oder Wasserstoffbrückenbindungen.

2.2. Instrumentation

Beckmann IR 4210 und IR 20 AX, 10 cm-Gaszelle UG-03, Referenzstrahlabschwächer AT-04, Spaltprogramm 0,3 mm bei 3000 cm^{-1}, 7 min über gesamten Bereich, Verstärkung 1,2.

*** Angaben über die Abkürzungen der benuzten GV, über die Lösungsmittel sowie die verwendeten Stink- und Riechstoffe vgl. Abschnitt 3.4.

3. GAS-CHROMATOGRAPHISCHE UNTERSUCHUNGEN

3.1. Langzeitversuch

Gasförmige Stinkstoffe und gasförmige GV beließ man während eines halben Jahres miteinander in Kontakt. In einem 0,5 L-Kolben wurden flache Schälchen mit 2,00 g Stinksubstanz und jeweils 3,50 g GV gebracht. Der Kolben wurde mit einem Septum verschlossen. Wöchentlich wurde eine Probe von 5 ml der Gasphase für die gas-chromatographische Untersuchung entnommen. Durch Nachverdampfung war die Stinksubstanz fast ganz verbraucht (Rest 0,24 g), die GV hatten kaum an Gewicht verloren (Rest von Produkt B***) 2,98 g; Rest von Produkt C***) 3,39 g). Die Gas-Chromatogramme, wöchentlich während des halben Jahres ermittelt, zeigten keinerlei Hinweise auf stattgefundene chemische Umsetzungen der in der Gasphase vorhandenen Stinkstoffe mit den GV.

3.2. Prinzip

Jede Beurteilung und quantitative Erfassung einer Geruchseliminierung ist letztlich nur in der Gasphase möglich. Wir wählten deshalb ein Verfahren, bei dem die Einwirkung der GV auf die gasförmigen Geruchsstoffe in Form eines Aerosols erfolgte. Dies entspricht auch der Praxis bei Anwendung von Raumsprayprodukten.

Mit Hilfe eines Gasspritze wurde eine bestimmte Menge gasförmiger Geruchsstoffe in eine luftgefüllte Headspace-Birne gebracht, um gas-chromatographisch als Nullversuch die quantitativen Verhältnisse festzustellen. Nach Einsprühen genau bemessener Mengen GV oder anderer Stoffe (mit Treibgas F 12 A abgefüllt; vgl. Abschnitt 3.5.) erfolgte eine weitere Probenahme für die Gas-Chromatographie. Ein Vergleich mit dem Nullversuch ergibt die relative Abnahme der Geruchsstoffe im Gasraum als Maß für die Wirksamkeit des GV.

3.3. Instrumentation

Gas-Chromatograph Hewlett-Packard, Modell Research 5750 G mit Doppelflammenionisationsdetektor. Um Zersetzung der Substanzen an Metallen auszuschließen (4), wurde eine Glassäule von 1,8 m Länge, einem inneren Durchmesser von 2 mm mit einer Füllung von 5 % Carbowachs 20 M auf Chromosorb WAW-DMCS 80/100 mesh benutzt. Da das Stinkstoff- und das Riechstoffgemisch in unterschiedlichen Temperaturbereichen sieden, wurde temperaturprogrammiert getrennt: das Stinkstoffgemisch von 50°—100 °C mit einer Heizrate von 8 °C/min, das Riechstoffgemisch von 100°—150 °C mit 10 °C/min. Die Vermessung der Peaks erfolgte durch angeschlossenen Integrator.

3.4. Versuchsmaterial

Bisher erschienene Firmenschriften über die jeweiligen GV weisen immer auf die Selektivität dieser Stoffe für organische Stickstoff- und Schwefelverbindungen hin, wie sie in Haushalt und Industrie vorkommen. Zur Untersuchung mischten wir Verbindungen mit unangenehmem Geruch zu einem (gasförmigen) Stinkstoffgemisch *(Tab. 1)*.

Tabelle 1

Verbindungen	Volumteile	Dampfdruck Torr (20° C)	Molekulargewicht
Diäthylsulfid	10	10 (—7,8 °C)	90,19
Butyronitril	30	26	69,11
Allylsenföl	20	2,54	99,16
Dibutylsulfid	40	1	146,30
	100 ml Gas ≅ 10 Vol. % in der Gasphase		

Diesem Stinkstoffgemisch wurde eine Mischung aus Stoffen verschiedener chemischer Gruppen, die in der Parfümerie Verwendung finden, als Riechstoffgemisch gegenübergestellt *(Tab. 2)*.

Tabelle 2

Verbindungen	Volumteile	Dampfdruck Torr (20° C)	Molekulargewicht
Limonen	20	—	136,22
p-Cresylmethyläther	20	—	122,16
Linalool	30	0,12	154,24
Benzylacetat	30	80	150,17
	100 ml Gas ≅ 28 Vol. % in der Gasphase		

Für die Auswahl waren Kriterien, die eine einwandfreie gas-chromatographische Handhabung ermöglichten, maßgeblich, z. B. unterschiedliche Retentionszeiten innerhalb eines engen Siedebereiches.

Die geprüften GV waren handelsübliche Präparate. Grillocin® HY 66 (abgekürzt: A) (5) wurde zu je 20 Gew.-% in Diäthylglykolmonoäthyläther [APV; Badische Anilin- und Sodafabrik (BASF), D-67 Ludwigshafen/Rh.], in 96 vol.%igem Äthanol bzw. in Solketal (2-Dimethyl-4-oxymethyl-1,3-dioxalan; Brenntag; D-433 Mülheim/Ruhr) gelöst. Metazene (abgekürzt: B) (6), ein flüssiges Produkt, das nach Herstellerangaben 80 Gew.-% Laurylmethacrylat und 20 Gew.-% geruchfreie Kohlenwasserstoffe und Inhibitor enthält,

wurde ohne Lösungsmittel eingesetzt. Das Produkt Sinodor (abgekürzt: C) (7) wurde als solches verwendet.

Die Blindversuche wurden mit 96 vol.%igem Äthanol, mit APV an sich, mit einer Lösung von 5 % Polyvinylpyrrolidon (PVP) in 96 vol.%igem Äthanol [Luviskol VA 37E; Badische Anilin- und Sodafabrik (BASF), D-67 Ludwigshafen/Rh.] und mit Solketal an sich ausgeführt.

Die Abfüllung erfolgte in diesen Fällen mit 50 Gew.-% Wirkstoff: 50 Gew.-% F 12 A. Von 1,2-Propylenglykol wurden 10 Gew.-% in F 12 A gelöst (siehe bemerkung in *Tab. 3)*.

3.5. Probenaufbereitung (Abb. 1)

Herstellung einer gesättigten Gasphase durch Schütteln der einzelnen Stink- bzw. Riechstoffe in Kolben (a). Überführung eines Teils der jeweiligen Gasphase (s. *Tab. 1* und *2)* in die 500 ml-Headspace-Birne mit Niveaugefäß (b), Sperrflüssigkeit Hg. Gasanteil des zusammengestellten Stinkstoffgemisches ≙ 10 Vol.%, bei Riechstoffgemisch ≙ 28 Vol.%. Einsprühen von 5 × 0,1 g F 12 A (Dosierventil) durch das Septum in die Headspace-Birne (c). Nach Gleichgewichtseinstellung (2 Std.) Entnahme von 5 ml für den gas-chromatographischen Nullversuch.

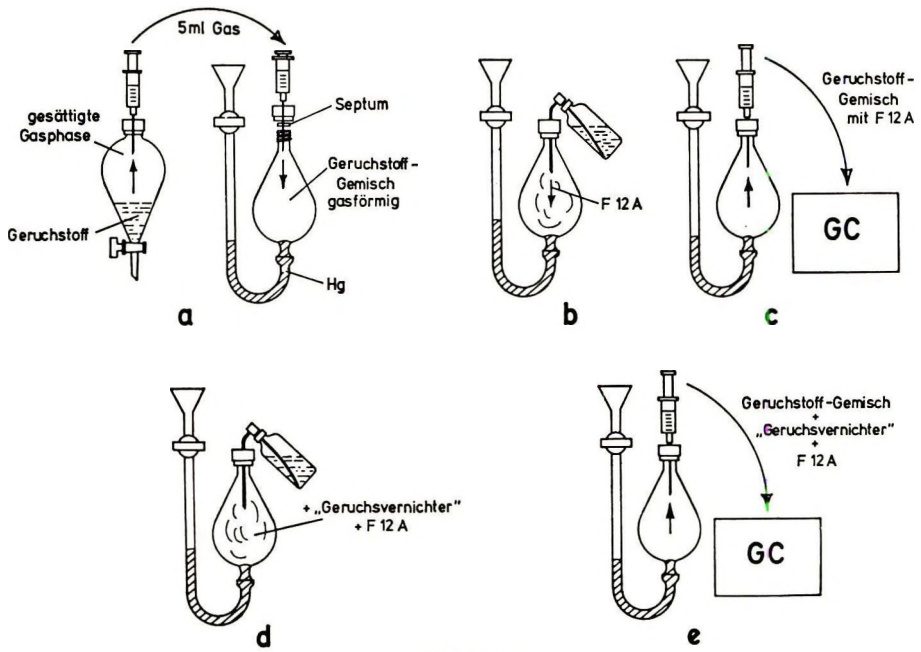

Abbildung 1
Schematische Darstellung der Versuchsanordnung

Tabelle 3

Ergebnisse von 7-Tage-Versuchen

Wirkstoff	Stinkstoffe	Flächenprozente vor dem Versprühen des Wirkstoffs	Flächenprozente 24—175 Stunden nach dem Versprühen des Wirkstoffs						
			24	30	45	55	70	95	175
Äthanol	Butyronitril I	16,64 ± 0,75	4,60	4,69	4,31	4,36	4,67	3,92	3,38
	Butyronitril II + Allylsenföl I	0,45 ± 0,05	0,01	0,00	0,00	0,00	0,00	0,00	0,00
	Allylsenföl II + Dibutylsulfid	1,34 ± 0,07	0,11	0,12	0,10	0,09	0,08	0,06	0,04
Äthanol + A	Butyronitril I	19,01 ± 1,03	3,96	4,96	4,31	4,95	5,10	4,96	3,37
	Butyronitril II + Allylsenföl I	0,33 ± 0,05	0,00	0,00	0,00	0,00	0,00	0,00	0,00
	Allylsenföl II + Dibutylsulfid	1,07 ± 0,03	0,02	0,01	0,00	0,00	0,00	0,00	0,00

Wirkstoff	Riechstoffe	Flächenprozente vor dem Versprühen des Wirkstoffs	Flächenprozente nach dem Versprühen des Wirkstoffs	relativer Auswascheffekt in %
Äthanol	Limonen	9,84 ± 0,72	1,18 ± 0,19	88
	p-Cresylmethyläther	4,78 ± 0,25	0,32 ± 0,14	93
	Linalool	7,05 ± 0,92	0,46 ± 0,27	93
	Benzylacetat	2,76 ± 0,34	0,34 ± 0,18	88
Äthanol + A	Limonen	7,35 ± 0,70	0,39 ± 0,02	95
	p-Cresylmethyläther	4,85 ± 0,26	0,13 ± 0,03	97
	Linalool	5,95 ± 0,64	0,13 ± 0,05	98
	Benzylacetat	4,13 ± 0,24	0,19 ± 0,07	95

Wirkstoff	Stinkstoffe	Flächenprozente vor dem Versprühen des Wirkstoffs	Flächenprozente nach dem Versprühen des Wirkstoffs	relativer Auswascheffekt in %
Solketal	Diäthylsulfid	9,19 ± 0,36	5,68 ± 0,57	38
	Butyronitril I	22,31 ± 0,72	7,59 ± 0,94	66
	Butyronitril II + Allylsenföl I	0,77 ± 0,04	0,14 ± 0,04	82
	Allylsenföl II + Dibutylsulfid	0,46 ± 0,04	0,17 ± 0,02	63
Solketal + A	Diäthylsulfid	10,65 ± 0,37	6,98 ± 0,23	35
	Butyronitril I	22,17 ± 0,33	8,66 ± 0,12	61
	Butyronitril II + Allylsenföl I	0,54 ± 0,03	0,04 ± 0,02	93
	Allylsenföl II + Dibutylsulfid	1,42 ± 0,01	0,25 ± 0,01	82
C	Diäthylsulfid	12,52 ± 0,45	7,51 ± 0,43	40
	Butyronitril I	21,92 ± 0,85	12,49 ± 0,65	43
	Butyronitril II + Allylsenföl I	0,67 ± 0,06	0,07 ± 0,03	90
	Allylsenföl II + Dibutylsulfid	0,41 ± 0,06	0,06 ± 0,00	85
B	Diäthylsulfid	—	—	—
	Butyronitril I	21,49 ± 1,41	12,59 ± 0,62	41
	Butyronitril II + Allylsenföl I	0,36 ± 0,04	0,00	100
	Allylsenföl II + Dibutylsulfid	0,85 ± 0,05	0,05 ± 0,02	94
1,2-Propylenglykol	Diäthylsulfid	10,43 ± 0,52	8,06 ± 0,13	23
	Butyronitril I	14,95 ± 0,68	7,81 ± 0,35	48
	Butyronitril II + Allylsenföl I	0,30 ± 0,03	0,08 ± 0,02	93
	Allylsenföl II + Dibutylsulfid	1,12 ± 0,08	0,32 ± 0,02	71
Da 1,2-Propylenglykol mit F 12 A nur als Emulsion vorliegt, ist eine gleichmäßige Nebelbildung nicht erzielbar. Deshalb der geringere Auswascheffekt.				

Wirkstoff	Stinkstoffe	Flächenprozente vor dem Versprühen des Wirkstoffs	Flächenprozente nach dem Versprühen des Wirkstoffs	relativer Auswascheffekt in %
Äthanol	Diäthylsulfid	— Fehler	— Fehler	— *
	Butyronitril I	33,86 ± 1,58	14,73 ± 1,08	57 **
		16,65 ± 0,84 ***	4,86 ± 0,25	71
	Butyronitril II + Allylsenföl I	0,47 ± 0,04	0,04 ± 0,02	92
	Allylsenföl II + Dibutylsulfid	0,33 ± 0,03	0,03 ± 0,01	91
Äthanol + A	Diäthylsulfid	—	—	—
	Butyronitril I	25,48 ± 0,39	7,00 ± 0,39	73
	Butyronitril II + Allylsenföl I	0,71 ± 0,04	0,02 ± 0,00	97
	Allylsenföl II + Dibutylsulfid	0,36 ± 0,03	0,00	100
APV	Diäthylsulfid	—	—	—
	Butyronitril I	26,23 ± 0,60	7,77 ± 0,57	71
	Butyronitril II + Allylsenföl I	0,70 ± 0,06	0,00	100
	Allylsenföl II + Dibutylsulfid	1,57 ± 0,04	0,04 ± 0,02	97
APV + A	Diäthylsulfid	—	—	—
	Butyronitril I	23,05 ± 0,37	6,20 ± 0,19	73
	Butyronitril II + Allylsenföl I	0,50 ± 0,03	0,00	100
	Allylsenföl II + Dibutylsulfid	1,26 ± 0,06	0,06 ± 0,03	95
Äthanol + PVP (Luvikol VA 37 E)	Diäthylsulfid	—	—	—
	Butyronitril I	27,13 ± 1,04	6,68 ± 0,52	75
	Butyronitril II + Allylsenföl I	0,68 ± 0,06	0,00	100
	Allylsenföl II + Dibutylsulfid	2,69 ± 0,11	0,16 ± 0,07	94

* Diäthylsulfild wird im Chromatogramm vom Lösungsmittel überlagert

** wegen erhöhter Ausgangskonzentration verringerter relativer Auswascheffekt

*** Konzentration der übrigen Versuchsreihe

Einsprühen von 0,5 g GV-Abfüllung oder anderer zu prüfender Stoffe *(Tab. 1* und *2)* mittels Dosierventil durch das Septum (d). Es ergibt sich ein Verhältnis von Geruchs- zu Wirkstoffen im Bereich von ca. $1 \cdot 10^{-6}$ zu ca. $5 \cdot 10^{-3}$ Mol, also etwa ein tausendfacher Überschuß der Wirkstoffe.

In abgestuften Zeiten wurden je 5 ml der Gasphase mittels Gasspritze zur gas-chromatographischen Kontrolle (e) entnommen.

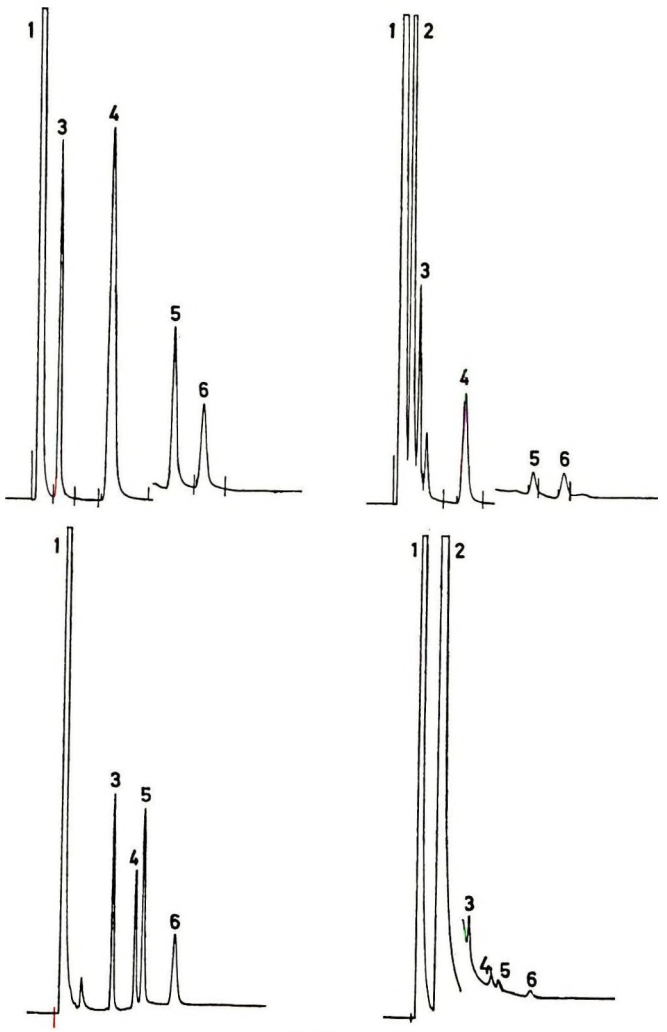

Abbildung 2

Obere Gas-Chromatogramme, links: Stinkstoffgemisch Nullversuch; rechts: Stinkstoffgemisch mit Solketal. 1 Treibgas F 12 A, 2 Lösungsmittel, 3 Diäthylsulfid, 4 Butyronitril I, 5 Butyronitril II + Allylsenföl I, 6 Allylsenföl II + Dibutylsulfid. — Untere Gas-Chromatogramme, links: Riechstoff Nullversuch; rechts: Riechstoffgemisch mit A in Äthanol. 1 Treibgas F 12 A, 2 Äthanol, 3 Limonen, 4 p-Cresylmethyläther, 5 Linalool, 6 Benzylacetat.

4. AUSWERTUNG DER GAS-CHROMATOGRAMME

Abb. 2 zeigt oben links das Chromatogramm des Nullversuchs mit Stinkstoffgemisch gemäß *Tab. 1*, daneben das zugehörige Chromatogramm nach Anwendung von Solketal, jeweils 2 Std. nach dem Einsprühen. Diese Chromatogramme stehen stellvertretend für alle Versuche mit Stinkstoffgemisch gemäß *Tab. 1.*

Die Chromatogramme darunter geben die Verhältnisse in der gleichen Art für das Riechstoffgemisch *(Tab. 2)* und nach Anwendung von A in Äthanol wieder. *Abb. 3* (Stinkstoffgemisch) und *Abb. 4* (Riechstoffgemisch) zeigen die Auswertung der Flächenprozente der angegebenen Peaks, die der Konzentration des jeweiligen Geruchsstoffes entsprechen. Somit ist die relative Abnahme des Geruchsstoffes in der Gasphase berechenbar.

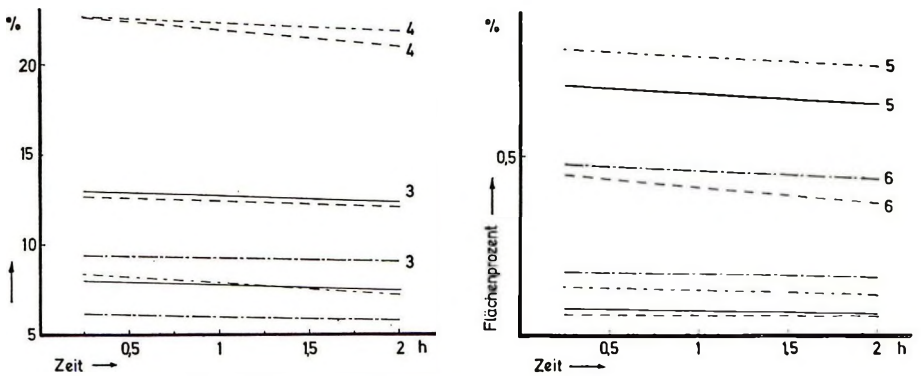

Abbildung 3

Linke graphische Darstellung: ——— Diäthylsulfid mit C (3 = Nullversuch), — — — — Butyronitril I mit C (4 = Nullversuch), —·—·— Diäthylsulfid mit Solketal (3 = Nullversuch), —·—·—·— Butyronitril I mit Solketal (4 = Nullversuch). — Rechte graphische Darstellung: ——— Butyronitril II + Allylsenföl I mit C (5 = Nullversuch), — — — — Allylsenföl II + Dibutylsulfid mit C (6 = Nullversuch), —·—·—·— Butyronitril II + Allylsenföl I mit Solketal (5 = Nullversuch), —·—·— Allylsenföl + Dibutylsulfid mit Solketal (6 = Nullversuch)

Die Berechnung erfolgt unter Berücksichtigung der Zunahme von F 12 A. Die anfänglich stärkere Abnahme der Stinkstoffe ist nur auf mangelndes Gleichgewicht (Abkühlung durch entspanntes F 12 A und noch schwebende Aerosoltröpfchen, die in die Spritze gesaugt werden) zurückzuführen. Die eingezeichneten Kurven sind alle durch Fehlerausgleichsrechnung begradigt.

Ein Langzeitversuch mit der Kombination Stinkstoffe + A in Äthanol und Stinkstoffe + Äthanol beweist, daß sich die Gaskonzentration auch nach 7 Tagen nur unwesentlich ändert *(Tab. 3).* Das bedeutet, daß in einem abgeschlossenen System die einmal aus der Gasphase entfernten Substanzen nicht mehr in die Gasphase zurückdiffundieren.

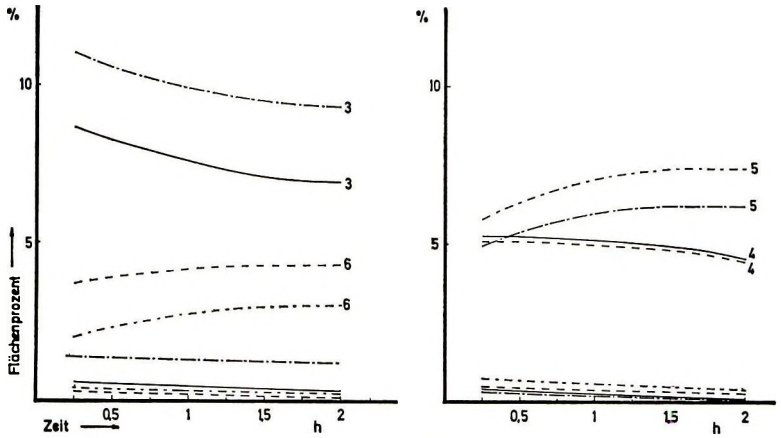

Abbildung 4

Links: ——— Limonen mit A in Äthanol (3 = Nullversuch), —·—·—· Limonen mit Äthanol (3 = Nullversuch), — — — — Benzylacetat mit A in Äthanol (6 = Nullversuch), — - — - — Benzylacetat mit Äthanol (6 = Nullversuch). — Rechts: ——— p-Cresylmethyläther mit A in Äthanol (4 = Nullversuch), — — — — p-Cresylmethyläther mit Äthanol (4 = Nullversuch), —·—·—· Linalool mit A in Äthanol (5 = Nullversuch), — - — - Linalool mit Äthanol (5 = Nullversuch)

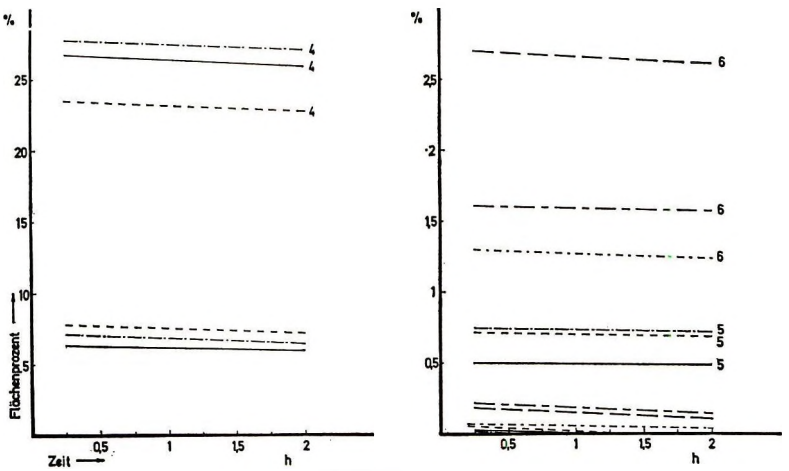

Abbildung 5

Links: — — — — Butyronitril I mit A in APV (4 im Nullversuch), —·—·—· Butyronitril I mit PVP in Äthanol (4 im Nullversuch), ——— Butyronitril I mit APV (4 im Nullversuch). — Rechts: ——— Butyronitril II + Allylsenföl I mit A in APV (5 im Nullversuch), — — — — Butyronitril II + Allylsenföl mit PVP in Äthanol (5 im Nullversuch), —·—·— Butyronitril + Allylsenföl I mit APV (5 im Nullversuch), — - — - — Allylsenföl II + Dibutylsulfid mit A in APV (6 im Nullversuch), — — — Allylsenföl + Dibutylsulfid mit PVP in Äthanol (6 im Nullversuch), ——— Allylsenföl II + Dibutylsulfid mit APV (6 im Nullversuch)

Abb. 5 zeigt eine Gegenüberstellung von Stinkstoffgemisch mit APV, A in APV und PVP in Äthanol.

Alle untersuchten Kombinationen gleichen in ihren Chromatogrammen einander prinzipiell. Es sind nur quantitative Unterschiede feststellbar (vgl. *Tab. 3*).

5. DISKUSSION DER ERGEBNISSE

Für die Versuche wurde eine wesentlich höhere Ausgangskonzentration von Geruchsstoffen und GV, als sie in der Praxis vorkommen, gewählt, um in einem für die GC-Analyse noch sinnvollen Bereich zu arbeiten. Unter den gegebenen Versuchsbedingungen lassen sich je nach Anfangskonzentration mit den sog. GV relative Abnahmen der Stink- und Riechstoffe in der Gasphase zwischen 50 % und 100 % erzielen.

Dies führt zu der

1. Feststellung: *Selektivität der sog. GV für „schlechte Gerüche" ist nicht gegeben.* Ein Vergleich der GV mit den geprüften Lösungsmitteln zeigt bezüglich der erzielbaren Konzentrationsverminderung identische Größenordnung. Äthanol und APV liefern Werte für die Konzentrationsabnahme, die über diejenigen für die GV C und B und etwa gleich mit A liegen. Somit ergibt sich als

2. Feststellung: Da man bei einem Zusammentreffen von Äthanol, APV und Solketal mit Geruchsstoffen *jede chemische Reaktion ausschließen kann,* man aber andererseits dieselben Abnahmeraten wie bei den sog. GV erzielt, ist zu schließen, *daß die Wirkung auf rein physikalischer Lösung beruht.* Es ist anzunehmen, daß der Auswascheffekt den bekannten physikalischen Gesetzen gehorcht und somit Dipolmoment, Polarisierbarkeit, Molekülgröße, Dampfdruck etc. der Stoffe beider Phasen eine Rolle spielen. Ein Lösungsmittel mit verhältnismäßig schlechtem Auswascheffekt (z. B. Solketal) läßt sich in seiner Wirkung durch Zusatz eines GV (z. B. A) nicht steigern.

ZUSAMMENFASSUNG

Die Beseitigung unangenehmer Gerüche bei Kosmetika, im Haushalt und bei technischen Produkten ist ein immer wiederkehrendes Problem. Die hierzu angebotenen Geruchsvernichter zeigen einen Effekt, von dem man vermutet, er beruhe auf chemischen Reaktionen. Es wird ferner vielfach behauptet, die Geruchsvernichter wirkten selektiv auf jene unangenehm riechenden Stoffe. Daher wurde geprüft, in welchem Maße Stink- und Riechstoffe bei Anwendung von Geruchsvernichtern aus der Gasphase beseitigt werden können und nach

welchem Mechanismus sich die Beseitigung vollzieht. Die äußerst schwierigen infrarotspektroskopischen Untersuchungen zum Nachweis chemischer Bindungen geben in keinem Fall einen Hinweis auf Molekül-, Ionen- oder Wasserstoffbrückenbindungen. Mit Versuchen, bei denen die Einwirkung der Geruchsvernichter auf die gasförmigen Geruchsstoffe in Form eines Aerosols erfolgte, wurde der Praxis bei Anwendung eines Raumsprays entsprochen. Die Abnahme der Geruchsstoffkonzentration in der Gasphase nach Anwendung von Geruchsvernichtern und bestimmten Lösungsmitteln wurde gas-chromatisch quantitativ bestimmt. Hierbei ergab sich, daß Selektivität der sogenannten Geruchsvernichter für „schlechte Gerüche" nicht besteht, die Geruchsminderung auf rein physikalischer Lösung, also auf einem Auswascheffekt beruht, mit bestimmten Lösungsmitteln dieselben Effekte erzielt werden können und geringfügig stärkere Konzentrationsabnahmen der Geruchsstoffe durch Filmbildner erfolgt, weil sie vermutlich sowohl schwebende Tröpfchen als auch Gravitationsniederschläge umhüllen und dadurch die Geruchsstoff-Moleküle fixieren.

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J. Soc. Cosmet. Chem., **26**, 247–254 (May 1975)

Isolation and Identification of the Protein Component of Hair Melanin

KATHLEEN HALL, B.Sc. and LESZEK J. WOLFRAM, Ph. D.*

Synopsis—ELECTRON MICROSCOPIC STUDIES have previously revealed that, in the MELANOCYTE, granules appear to exist in varying stages of development. They represent preliminary synthesis of NONMELANIZED GRANULES and are composed of coiled protein filaments or membranes. This protein matrix serves as a substrate for subsequent deposition of the melanin polymer and thus becomes totally encapsulated by the pigment. Upon reaching complete melanization, the granules appear uniformly dense, and no internal structure can be resolved by conventional techniques.

Previous attempts to identify the MELANO-PROTEIN have been hindered by the chemical inertness of the melanin capsule. A technique for solubilizing the melanin was found and provides a facile method of isolating the protein moiety present in the pigment granule. The chemical composition of this protein has also been determined.

I. Introduction

The site of melanin formation in vertebrates is the melanocyte. This is a specialized, distinctive cell characterized by two or more dendritic processes and usually located in a region adjacent to the developing cells endowed with the function of pigment transfer. In the case of hair follicles, the melanocytes are normally found dispersed among the rapidly dividing cortical cells of the hair bulb at the apex of a small cavity known as the dermal papilla. Pigment granules formed in these melanocytes are transferred to the cortical cells of the forming hair, and during the process of extrusion and keratinization the melanin pigment becomes aligned to the axis of the fiber.

The fine structure of both melanocytes and melanosomes has attracted much attention. The pioneering work of Birbeck (1), Drochmans (2), and Moyer (3) has demonstrated the developmental changes in the elaboration of black pigment granules. Two main successive steps in the formation of a

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melanosome can be represented as follows: first, the formation of a protein matrix composed of coiled filaments or membranes; second, the deposition of melanin polymer onto the protein matrix. As the melanin deposition continues, the membranous support becomes so obliterated that the granules appear uniformly electron dense and devoid of any structural detail. From an investigative point of view, complete melanization results in somewhat of a paradox. The known resistance of the melanin polymer to chemical reagents allows for easy separation of the pigment and its encapsulated protein from the fibrous keratin, but at the same time effectively prevents the isolation of the proteinaceous matrix from its melanin environment. Several attempts have been made to extract the protein component from the matrix granules. Thus Serra (4) claims to have isolated a melano protein complex free from fiber contamination. However, his high values for protein content of the pigment (over 50%) appear incompatible with the available elemental analysis of the composite materials. Laxer (5), who had isolated the pigment from hair by treatment of the latter in phenol hydrate-thioglycollic acid mixtures, refluxed the melanin granules with 6*N* HCl for extended periods of time (>48 hours) and analyzed the hydrolysate by paper chromatography. Most of the amino-acids present in the keratin were found in the hydrolysate, but no quantitative determinations were made.

Recent investigations by Gjesdal (6) have confirmed the presence of protein in pigment granules isolated by hydrolyzing variously colored fibers with dilute alkali. Again, a quantitative analysis was not attempted, and Gjesdal noted that the protein present in the pigment is strongly bound within the granule.

In the course of our work on hair bleaching we have developed a very mild, yet effective procedure for solubilization of the melanin pigment (7). It appeared to us that this method offers a unique way for a very facile separation of the protein-bound pigment. The utility of such an approach is the subject of this paper.

II. Materials and methods

A. *Poodle Hair*

Black poodle hair was obtained from random samples of hair clippings. The hair was purified by Soxhlet extraction with methylene chloride followed by absolute methanol for four hours each. The hair was then rinsed well with deionized water and allowed to dry.

B. *Oriental Hair*

Samples of Oriental hair, approximately 5 in. in length, were obtained from an individual known to have untreated hair. The distal ends of the fibers were trimmed and discarded; the remaining hair was purified by the extraction procedure described above.

C. *Hair Melanin*

The melanin was isolated by careful dissection of the ink sacks, previously hair by the nonhydrolytic method of Laxer (5). The hair sample was refluxed for 24 hours with a phenol hydrate-thioglycollic acid mixture (PHT). The pigment was separated by centrifuging the solution in cellulose nitrate tubes at 1800 rpm for 45 min. The melanin was then washed with two successive portions of PHT, rinsed several times with 40% aqueous ethanol, then dried *in vacuo* at 60°C.

D. *Squid Melanin*

The melanin was isolated by careful dissection of the ink sacs, previously removed from approximately 1 lb of squid. The pigment was rinsed several times with water, then soaked in 6*N* HCl for 24 hours at ambient temperature. Removal of the acid was accomplished by thorough water rinsing, and centrifugation of pigment followed by air drying.

E. *Chemical Reagents*

Commercially available, reagent grade solvents were utilized in this study without further purification.

F. *Amino-acid analysis*

Amino-acid analysis was performed on the Phoenix M7800 automatic amino-acid analyzer.

III. Results and Discussion

We have recently shown (7) that the bleaching of melanin by aqueous solutions of H_2O_2 involves two successive processes: solubilization of the pigment granules followed by decolorization of the solubilize melanin. The reaction can be restricted to the solubilization stage alone if dilute solutions of H_2O_2 are used. Under such conditions, the polymeric character of the pigment is retained, and the extent of oxidative degradation of the protein component is marginal. The molecular weight of the solubilized pigment was calculated to be 11,400 and 15,000 via osmotic pressure measurements and thin-layer gel filtration, respectively (7).

The solubilized melanin was prepared as follows: 100 mg of the pigment was suspended with stirring in 20 ml of 1% hydrogen preoxide at pH 10 (ammonia was used for pH adjustment) and 25°C. Complete dissolution of the pigment took place within 60 min. At this point the excess peroxide was destroyed by platinum black, and the product was lyophilized yielding a black, highly lustrous water-soluble material. It is of interest to note that the solubility characteristics of the oxidized melanin could be changed by esterifica-

tion of its carboxylic side chains by treatment in methanol/HCl. This methylated melanin was now water insoluble under acidic and neutral conditions but dissolved readily at pH 9 and above as the groups of the hydroxyindole residues began to ionize.

The solubility characteristic of the solubilized melanin thus appeared to be controlled by the ionization of the carboxylic side chains present in this melano-protein moiety. Aqueous solutions of the latter were found to form copious precipitates upon acidification below pH 4. Although both the melanin and the protein were present in the precipitate, only the protein was found in the supernatant. The melanin-free protein present in the supernatant accounted for 23% of the total protein associated originally with the pigment. Complete separation of the protein component was attained by acid hydrolysis of the combined fractions, i.e., the precipitate and supernate in 6*N* HCl for 16 hours at 105°C. Table I lists the amino-acid content of the protein component of the melanins obtained from human hair as well as from dog hair. For comparison, the amino-acid composition of the corresponding hair keratin is also given.

It is clearly evident that the chemical composition of the melano-protein differs greatly from that of the keratin. Particularly striking is the high content of basic amino-acids present in the former. Although the extent of amidation of the glutamic acid is unknown, it is tempting to suggest that the protein, in its native state, is positively charged and thus offers an ideal site for the firm embedding of negatively charged melanin precursors and their polymers. This way, the cohesion of alternate protein-melanin layers is greatly enhanced, and thus the electrostatic interaction may be the instrumenal factor in the initial stages of formation of high-density pigment granules.

The melano-protein contains two acids which have not been found as the components of keratins. These are taurine and β-alanine. Although these compounds might be by-products of the solubilization process, the possibility that they are associated with the biosynthesis of the melanin should not be overlooked.

The protein content of the dog hair melanin, calculated from the amino-acid data, accounts to 7.8%, based on the weight of the solubilized pigment. A similar value of 9.4% was obtained for the melano-protein content of melanin which was isolated from Oriental hair. These appear to be reasonable values. The solubilizing treatment, although effective in bringing about physical destruction of the pigment granules, is not likely to cause an extensive oxidative degradation of the encased protein. The chemical attack in this case appears to be confined to the residues of sulfur-containing amino-acids. Evidence for the latter is supported by chemical analysis of extensively bleached hair; in this case the reaction between keratin and hydrogen peroxide is mainly confined to the cystine cross-links giving rise to the formation of cysteic acid residues. Thus the cysteic acid found in large quantities in the melano-

Table I

Amino-Acid Composition of the Protein Isolated from the Melanin of Oriental Hair and Poodle Hair

Type of Side Chain and Amino Acid	Melano-Protein μM/g	Oriental Hair μM/g	Melano-Protein μM/g	Poodle Hair μM/g
Aliphatic	2656	1963	2755	1962
Glycine	1005	455	1143	572
Alanine	459	365	504	392
Valine	478	448	372	386
Isoleucine	210	207	162	184
Leucine	466	488	500	428
β-Alanine	38	...	74	...
Aliphatic hydroxyl	967	1425	747	1396
Threonine	417	533	342	552
Serine	550	892	405	844
Aromatic	276	288	346	299
Tyrosine	101	159	129	166
Phenylanine	175	129	217	133
Acidic	1619	1407	1579	1256
Aspartic acid	682	427	559	428
Glutamic acid	937	980	1020	828
Basic	1037	771	1507	518
Lysine	311	211	700	152
Histidine	156	71	230	48
Arginine	570	489	577	318
Sulfur containing	787	1391	521	1469
Cysteic acid	463	41	337	42
Taurine	36	...	14	...
Methionine sulfoxide	22	...	...	...
Methionine sulfone	184	...	101	...
Half-cystine	45	1312	42	1370
Methionine	37	38	27	56
Heterocyclic	453	600	358	601
Proline	453	600	358	601

protein is partially accounted for as a result of the oxidation of cystine occurring during the solubilization process. However, the bulk of the cysteic acid is not likely to arise from the mild oxidative treatment employed. Some support for this is obtained by the presence of cysteic acid in the hydrolyzates of intact melanin. The possibility of cysteic acid formation via disproportionation of products effected by the redox system of melanin's quinonoid structure is also rejected. A known quantity of cystine was added to solubilized melanin samples prior to hydrolysis; no increase in the cysteic acid content was detected.

Whether the disulfide bond of cystine is also the covalent link between the melanin polymer and the protein component of the granule is not known at this stage. We would like to point out, however, that the solubilization treatment does release some of the protein from its melano-protein moiety. As much as one-third of the total protein content can be separated from the melanin polymer by mere acidification of the solubilized pigment. The recently introduced technique of enzymatic hydrolysis (8) may prove to be an invaluable tool in determining both the frequency and the nature of melanin-protein covalent bonding.

A third melanin, a sepia melanin, was isolated from the squid ink sac and solubilized in the same manner previously described. This melanin exists as electron dense spheres measuring 0.1 to 0.3 μm across (9); the melanins isolated from the hair keratin were in the form of discrete granules, approximately 0.35 by 1.0 μ. Despite the physical difference and origin of the squid melanin, a similarity in the melano-protein compositions was observed for these melanins.

Calculation of the protein content of the squid melanin based on the data in Table II gave the value of 4.3%, a somewhat lower value than the one obtained in the case of the hair melanins examined. The basicity of the squid melano-protein was considerably less than in the corresponding proteins isolated from poodle hair melanin and Oriental hair melanin. This may be due to the need for more acidic functions within this particular structure in order to aid in the dispersion of the melanin ink in aqueous media. A comparison of the analytical data given in Tables I and II reveals that in many cases, either the concentrations or proportions of the respective amino acids are in close proximity. This is in spite of the diverse sources of these three proteins and possibly connotes that a chemically specific protein matrix is a prerequisite for the formation of melanin granules, irrespective of their animal origin. Such a hypothesis of protein specificity is new, and in view of the scarce experimental evidence available (namely, the chemical composition of 3 melano-proteins), it may be classified as speculation. Yet it might be the key to the activity of the melanosomes and the rate at which the melanin polymer is formed within these cells.

Table II

Amino-Acid Composition of the Protein Isolated from Squid Melanin

Type of Side Chain and Amino Acid	Melano-Protein μM/g
Aliphatic	2669
Glycine	975
Alanine	461
Valine	352
Isoleucine	327
Leucine	526
β-Alanine	28
Aliphatic Hydroxyl	462
Threonine	228
Serine	234
Aromatic	623
Tyrosine	237
Phenylalanine	386
Acidic	2002
Aspartic acid	1158
Glutamic acid	844
Basic	887
Lysine	240
Histidine	144
Arginine	503
Sulfur Containing	663
Cysteic acid	287
Taurine	163
Methionine sulfoxide	...
Methionine sulfone	139
Half-cystine	nil
Methionine	74
Heterocyclic	400
Proline	400

IV. Conclusion

The technique of melanin solubilization was successfully used in this investigation to elucidate the nature of proteins associated with pigments. There is no reason why this technique could not be extended to examine melanins of various origins to assert or reject our postulate of protein-matrix specificity.

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A Critical Comparison of Two Procedures for Antiperspirant Evaluation

W. M. WOODING, B.Ch.E.° and PAUL FINKLESTEIN, Ph. D.†

Presented December 2, 1974 New York City

Synopsis—This paper is concerned with EXPERIMENTAL DESIGN and DATA ANALYSIS PROCEDURES associated with GRAVIMETRIC AXILLAR ANTIPERSPIRANT TESTS. The most widely known of these tests requires the estimation of "control ratios" for each subject, based upon data obtained prior to the evaluation of product application results. These ratios are subsequently employed as correction factors to modify similar ratios in sweating responses, which are calculated during the product evaluation phase of the test. It will be shown that an improved procedure, which has been in use by this laboratory for more than 7 years, is substantially more economical with regard to time and effort than the above procedure. The new procedure does not require preliminary testing or the establishment of "control ratios." In addition, it is conceptually more rigorous, allows more definitive conclusions to be drawn, and conforms to established principles of statistical design and analysis.

In summary, it is claimed that the new method is easier, faster, more economical, and can be shown to be unbiased. The use of the two procedures will be illustrated with examples.

Introduction

Although several methods have been used to estimate the effectiveness of antiperspirants in the axillae, the gravimetric procedure is the most widely practiced one. This involves collection and weighing of axillar sweat under controlled conditions. It has the advantages of simplicity and of applicability to fairly large panels of human subjects and gives quantitative results suitable for mathematical analysis.

The earliest literature dealing with gravimetric procedures seems to be the well-known Fredell and Read paper, which was published in 1951 (1). These

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† Health Care Division, Johnson & Johnson, Inc., New Brunswick, N.J. 08901.

workers appear to have been the first to introduce the concept of using a t material in one axilla of each subject while leaving the other untreated a control. For a period of time before the initial use of the actual test materi they made daily sweat collections from both axillae of each subject taking p in the test. They then calculated pretest ratios (i.e., milligrams of sweat fr the right axillae—milligrams of sweat from the left axillae) for each su ject. These pretest or control ratios were subsequently compared with postt right/left ratios, the magnitude of the differences being taken as indicative the degree of efficacy of the antiperspirant material under test. Fredell a Longfellow in 1958 (2) suggested standardization of some of the test con tions, but continued to use right/left ratios in the same way. However, me ods of calculation were not clearly defined, and although several pretest terminations were recommended, the number was not made explicit. Also 1958, Daly (3) described a ratio method of calculation, but his procedure v not clear.

Laboratory protocols used by most workers today are similar to those scribed by these early workers, but the ratio which now appears to be univ sal is that of milligrams of sweat from treated axilla per milligrams fr control axilla, regardless of the side treated. The pretest ratio in this case, course, would be that of milligrams yielded by the axilla destined to treated per milligrams yielded in the opposite axilla. The method of adju ing posttest ratios with the pretest ratios has been described by Majors a Wild (4). The methods used by the older workers employing right/left rat were not entirely clear.

In the literature, we have been unable to find a description of the evolut of the modern axillar evaluation procedure using ratios, but protocols for te of antiperspirant with control, as well as the computation and use of trea side/control side ratios, are discussed in the Majors and Wild paper (4).

One of the authors (5) has described the analysis of gravimetric data c lected in experiments resembling those discussed by Majors and Wild, b without the use of pretest ratios. In that paper, certain aspects of the popu tion distributions of axillar milligram data were discussed, and a statisti model was proposed. Although the differences between that procedure a the ratio method do not seem to have attracted much attention, we believe possible to show conclusively that that method is superior to the ratio meth on the bases of statistical rigor, ease of correct computation, simplicity, a economy.

The purposes of this paper are as follows:

1. to propose a standard method for comparing an antiperspirant with control, which does not require the use of pretest ratios, and which will g statistically correct and unbiased estimates of per cent reduction and con dence intervals;

2. to show that commonly used data analysis methods, involving the det

mination of pretest ratios and the adjustment of posttest ratios thereby, are incorrect and produce incorrect results;

3. to demonstrate a statistically correct method of analyzing ratio data;

4. to show that point estimates of per cent reduction obtained with the ratio model disagree with those of the standard method, even when correct calculations are used; and

5. to briefly describe experimental designs for comparing more than one antiperspirant with control, using tests requiring 5 days, without the use of pretest ratios.

Experimental Procedure

For purposes of identification we will call our method the Sides Subjects Effects Model (SSEM) and the ratio method the Ratio Model (RM). As far as we can determine, we are the only workers consistently using the SSEM. Minor variations in the clinical procedures employed exist among users of the RM, and our description below is typical of, but not necessarily identical with, those used by a given investigator.

Both methods are concerned with the same problem: the comparison of an antiperspirant material with a control in order to assess efficacy; and obtaining this information as precisely as possible, without interference or distortion due to known "natural" differences between sides or among subjects (Majors and Wild have produced numerical evidence of a commonly hypothesized disparity between quantities of sweat produced in right and left axillae, which may be related to whether a subject is right or left handed (4)).

For simplicity, we will confine all of our detailed comparisons of the methods to "two-sample" tests in which one antiperspirant material is compared with control, although there is no theoretical reason why more than one antiperspirant may not be used, and designs of this kind will be described briefly in a later section.

The two methods are essentially identical in the clincal procedures used, with the exception of the use of pretest control runs in the RM; they may vary in some details, however. The object of both methods is to produce a point estimate of per cent reduction for antiperspirant relative to control, with confidence limits as a measure of its precision.

The SSEM procedure is done as follows. First 36 subjects are usually used. They are required to abstain from the use of any antiperspirant product for at least 4 weeks before the test begins, although they may use deodorants. The subjects are arbitrarily numbered before the test begins; then, using a table of random numbers (or a set of random numbers generated by a computer), 18 are randomly assigned to receive antiperspirant in the left axilla and control in the right, leaving the other 18 assigned to the opposite configuration. "Control" usually implies no treatment rather than a placebo. Applications of product are made daily for 4 days. Methods of application are stan-

dardized in detail, but depend upon the physical form of the antiperspirant material used. When both "ambient" and "hot-room" tests are to be run, the ambient test is done on the fourth day, beginning 1 hour after the fourth application of the product. A preweighed "ambient" pad of non-woven fabric is placed in each axilla of each subject using a harness device or tape to hold it in position. The subjects go about their normal business for 3 hours, then return to the clinic, where the pad is removed, reweighed, and the weight increase recorded. The subjects return to the clinic on the fifth day, receive a fifth application of the product and 1 hour later enter a hot room maintained at 105°F and 50% RH. They are seated in a random spatial order around the hot room, with a nonwoven fabric pad inserted in each axilla. They are asked to sit quietly with their hands in their laps and both feet on the floor throughout the hot-room period. After 40 min., the pads are removed and discarded (pad A). A second pad, this time preweighed, is then inserted (pad B), left in place for 20 min., then removed, reweighed, and the weight gain recorded. Finally, a third pad (pad C) is inserted and handled in the same manner as pad B. This completes the test, and the subjects are dismissed.

A number of details are not included in the above account, such as exact timing, randomization of the order of treating axillae, etc.

In the RM method, the procedures are similar, but one, two, or more days of pretest control data are obtained before treatments with antiperspirant begin. Confining the discussion to hot-room tests only for simplicity, these pretest runs are made both with pad B and pad C. It should be remembered that there are many variations of the RM test. Some conduct a test very similar to the SSEM just described. Others treat all subjects on one side, wait 2 weeks, then treat all on the other side. These variations, however, are variations in the experimental design and are unrelated to the central question to which we address ourselves. Therefore, to demonstrate differences between the two models on an equal basis, we will assume that both protocols are identical except, in the case of the RM, for the provision for the use of pretest ratios.

Statistical Considerations

General

The correct computational procedures for either model are dictated within rather narrow limits by the nature of the experimental design (including type of randomization) and the procedure of the protocol. Unless these particular computations are done, incorrect values of per cent reduction and/or confidence limits will be obtained. The computational procedures are therefore as important as the clinical ones and must follow valid statistical principles, take careful account of all underlying assumptions, and be compatible with the nature of the randomizations used in the experiments. These requirements

should be noted, as the use of incorrect randomization procedures, and computations are very common.

The procedures to be described must be applied separately to data from B and C pads. Because of the probable lack of independence of the errors of the two sets of data, it is not correct to regard them as replicates. Of course, averages may be used, if desired, or a slope analysis done. We prefer to analyze them separately. Actually, it is not necessary to use two pads, as we have found very close agreement between them in hundreds of tests.

SSEM Analysis (Crossover)

There is more than one possible model that could be adopted for the SSEM. The one we use at present, as reflected by the randomization procedures employed in assigning treatments to subjects and the subsequent handling of the subjects, is a crossover design (unlike many crossovers, however, the rows of the design represent sides rather than time periods). This design is illustrated in Figure 1. The columns represent subjects, the two rows sides, and the letters T_1 and T_2 the antiperspirant and control treatments. The statistical population model describing this design and its analysis is

$$y_{ijk} = \mu + \alpha_i + \sigma_j + \tau_k + \epsilon_{ijk}$$

where

y_{ijk} = ln mg of sweat for axilla i, subject j, and treatment k
μ = general mean
α_i = axilla i $i = 1, 2$
σ_j = subject j $j = 1, 2, \ldots n$
τ_k = treatment k $k = 1, 2$
ϵ_{ijk} = error for axilla i, subject j, and treatment k

α fixed; $\Sigma\alpha_i = 0$
σ random; $\sim N(0, \sigma_e^2)$, independent
τ fixed; $\Sigma\tau_k = 0$

As shown in the above model, the usual statistical assumptions underlying the design and analysis are implied: the errors are independent, randomly and normally distributed and homogeneous; treatments and sides represent fixed populations. The data analysis equates certain interactions with error under the assumption that they do not represent "real" effects in the population, as is done in Latin square designs. The analysis of data under this model follows that outlined briefly in a previous paper (5). It is assumed that the data are tabulated to show milligram values identified by subject, side, and treatment. The purpose of the analysis is to obtain estimates of the "treatments effect" and associated error uncontaminated by the influence of either the sides or subjects effects. Details of the analysis will be given later in the form of an example. Its major feature is that it requires transformation of the original milligram values to their logarithms before the statistical computa-

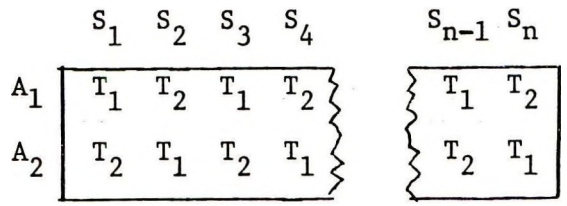

Figure 1. SSEM design represented as a crossover: A_1 represents left axillae; A_2 represents right axillae; S_1, S_2 . . . S_n represent subjects; T_1 represents antiperspirant; T_2 represents control. (Appropriate randomization not shown.)

tions begin in order to provide conformance to the assumptions of normality and homogeneity of variance. (The work reported in the earlier paper (5) showed this to be necessary, and has been confirmed by a large number of subsequent experiments.) A second important feature is the type of randomization procedure used, which is appropriate to the crossover design.

RM Analysis

The SSEM analysis, coupled with correct experimental design and randomization, can be shown to produce statistically unbiased estimates of per cent reduction and experimental error. The RM uses a similar design, but the analysis normally used assumes that sides effects are removed by the adjustment procedure. For the RM to remove side effects fully, however, it would be necessary that the pretest ratios be constants.

It is easy to observe by examination of any set of pretest ratios done repeatedly on the same subjects (see Table I) that the ratios are not constants. The fact that they are more uniform than milligram values of sweat produced is irrelevant, since ratios, not milligrams, are used in the adjustment procedure.

As an indicator of the degree of variability of pretest ratios with time, a correlation coefficient is a suitable statistic, although certain kinds of bias will remain undetected thereby. We carried out such tests on a number of pretest ratios determined 1, 3, and 21 days apart with the same subjects, using a rank correlation procedure to avoid violation of the statistical requirements of normality and homogeneity of variance. We obtained values ranging from less than 0.50 to 0.87 (a value of 1.00 would have indicated perfect correlation between successive measurements on the same subjects).

In addition to the above, we noted that the variance of adjusted mean posttest ratios is a function of the number of pretest measurements made and averaged, which are then used in the adjustment procedure. It is possible, with the use of a sufficient number of pretest measurements, to exercise considerable control of the experimental error of the final mean ratios. In one case, for example, the width of the confidence limits about the mean per cent reduction

Table I
pical Set of Two-Sample Test Data: Pad B Only, Four Applications before Posttest Readings (Milligrams and Ratios)

		PRETEST DATA				POSTTEST DATA
		Day 1		Day 2		(after 4 applications)
bject Number	Treatment On (side)	Right	Left	Right	Left	Right Left
13	R (mg)	606	599	690	704	263 630
	(ratio)	1.012		0.980		0.417
14	R (mg)	657	606	776	695	445 670
	(ratio)	1.084		1.117		0.664
15	R (mg)	630	555	646	593	304 380
	(ratio)	1.135		1.089		0.800
16	R (mg)	356	262	415	310	217 420
	(ratio)	1.359		1.339		0.517
17	R (mg)	400	409	489	546	336 497
	(ratio)	0.978		0.896		0.676
18	R (mg)	210	350	394	556	288 557
	(ratio)	0.600		0.709		0.517
19	L (mg)	789	332	1060	809	850 365
	(ratio)	0.421		0.763		0.429
20	L (mg)	710	589	750	568	400 257
	(ratio)	0.830		0.757		0.643
21	L (mg)	725	607	825	684	460 261
	(ratio)	0.837		0.829		0.567
22	L (mg)	809	663	312	243	430 200
	(ratio)	0.820		0.779		0.465
23	L (mg)	587	612	745	860	788 325
	(ratio)	1.043		1.154		0.412
24	L (mg)	618	461	547	523	555 283
	(ratio)	0.746		0.956		0.510

ıried, as the number of pretest measurements used to compute a geometric ean pretest ratio was increased, as follows:

Number of Pretest Measurements	Width of 95% CL about Per Cent Reduction Computed From Adjusted Posttest Ratios
1	26.4 (% reduction units)
2	22.6 (% reduction units)
4	17.2 (% reduction units)

This reduction of the confidence limits about the PR is sufficient so that, ven with geometric means of only two pretest ratios, it is often possible to qual or exceed the precision obtained with the SSEM when a single posttest etermination is used with the latter. This was the case in examples 2, 3, 4, and of Table VII (as will be shown later). Of course, the precision of the SSEM

can likewise be controlled by additional replication (for example, by averaging data obtained on successive days or by the use of additional test subjects*).

Aside from the above, the RM consistently yields different point estimates of per cent reduction than those obtained with the SSEM, using the same posttest data. Since the latter can be shown to be correct due to the balance of the experimental design, the question arises as to the source of the disagreement. Either both estimates are correct, or the use of the RM introduces a bias into the estimates. Since the confidence intervals obtained by either method (with correct calculations) generally include both point estimates, this question cannot be answered with certainty without further investigation. It seems clear, however, that the safe procedure is to use the SSEM, in view of its known validity (in addition to its practical advantage of requiring less time and effort).

The above remarks apply to the comparison when statistically correct methods are used in analyzing RM data. However, there appear to be as many methods of data analysis as there are practitioners, and all of those we have examined are incorrect and produce incorrect results. There are three common errors, which are (1) lack of recognition of the nonnormal character of the milligram-weight ratios and per cent reduction values; (2) lack of correct randomization prior to and during the clinical work; and (3) the use of a design implying a model not reflected by the analysis. The first of these errors can be remedied by an appropriate transformation of the ratios. Since it has been shown that the milligram weights used to form the ratios are log normally distributed (5), it follows that the adjusted ratios are also log normally distributed. Thus the proper transformation of the ratios is logarithmic. The problem of finding an appropriate transformation in order to validate the assumptions underlying the ordinary statistical procedures is a very common one and has been treated extensively in the literature of applied statistics (6–12).

The second error can be corrected by the use of the appropriate randomization procedures and the third by the performance of a suitable analysis.

Summary

Since the removal of the sides effect from error is a property of the analysis used with the SSEM and since the balance in the design guarantees an unbiased estimate of the per cent reduction, questions inherent in the use of the

*If this is attempted, however, care must be taken to do the analysis correctly, as measurements on the same subjects on successive days are likely to be correlated.

RM are easily avoided by using the SSEM. We will now present examples to illustrate the calculations for the SSEM in detail and to compare these with RM calculations.

Examples

General

Using data from several sources, we have analyzed 5 sets of two-sample data in three different ways. None of these methods will efficiently detect interactions among sides, treatments, and subjects, although a new design, discussed briefly later on and now being studied in this laboratory, has this capability. The most interesting of these interactions would be those involving treatments, of course, but it is not known at present whether they exist and, if so, whether their magnitudes are important.

We will illustrate the following procedures in this section.

A. SSEM procedure: This is the recommended analysis and gives unbiased estimates of per cent reduction. In this analysis, the effects of sides, subjects, and treatments are removed from the error estimates used to compute confidence intervals. The method does not require pretest ratios.

B. RM procedure (incorrect numerical analysis): This illustration will use one of the several incorrect procedures commonly employed to obtain per cent reduction estimates and confidence intervals, using adjusted ratios. The procedure is included to illustrate the distortion of results brought about by its violation of the statistical assumptions of normality and homogeneity of variance, as well as its failure to take residual sides effects into account.

C. RM procedure (correct analysis): This illustration will show the analysis of adjusted ratios when the above assumptions and a possible residual treatments times sides effect are accounted for. Its disagreement with the correct results yielded by the SSEM analysis (see (A) above) will be shown.

We shall describe the computations for each of the above procedures in some detail, using a single small set of data to allow the reader to compare the arithmetic involved in each case. We shall then summarize the results we obtained in applying the same procedures to each of the four other sets of similar two-sample data.

Table I gives a set of milligram data from 12 subjects, using the design of Fig. 1, with both pretest and posttest weights, for an antiperspirant known to be effective. For simplicity, we present data from only 1 pad and 1 hot-room collection posttest, the latter done after 4 antiperspirant applications. Note that, as was explained earlier, SSEM tests done for this laboratory normally use only 1 day of hot-room testing, done after 5 daily antiperspirant applications, and our test is completed in 5 days.

The data of the example represent a portion of a larger study, which included more than 1 active product. As was indicated earlier, the recommended minimum number of subjects for the SSEM procedure is about 36.

A. *SSEM Calculations*

1. For the SSEM calculations, the milligram posttest data are first transformed to their natural logarithms, which are shown in Table II.

2. An analysis of variance (ANOVA), mixed model (treatments and sides fixed), is done on the transformed data of Table II, using the crossover model. The ANOVA is shown in Table III, with back-transformed mean milligrams for treatment and control.

Table II
Natural Logs" of Posttest Data of Table I

Subject Number	Treated On	Right	Left
13	R	5.572	6.446
14	R	6.098	6.507
15	R	5.717	5.940
16	R	5.380	6.040
17	R	5.817	6.209
18	R	5.663	6.323
19	L	6.745	5.900
20	L	5.991	5.549
21	L	6.131	5.565
22	L	6.064	5.298
23	L	6.669	5.784
24	L	6.319	5.645

"Rounded to nearest three decimals for convenience; the actual analysis by computer used ten. It is generally sufficient to use two or three places if hand calculations are done.

Table III
ANOVA of Data of Table II

SOURCE OF VARIATION	DF	SS	MS	F	P
Treatments (Product versus control)	1	2.2795	2.2795	107.477	<0.0001
Sides (Left versus right)	1	0.0385	0.0385	1.816	<0.20
Subjects	11	1.0611	0.0965		
Error	10	0.2121	0.0212		
TOTAL	23	3.5912			

	Means of 12 Data	
	Based upon transformed data	Back-transformed to mg (antilogs)
Treatment	5.6656	288.8
Control	6.2820	534.9
Left axillae	5.9338	377.6
Right axillae	6.0139	409.1

3. The mean per cent reduction is computed as follows:

$$PR = \frac{\bar{T}_2 - \bar{T}_1}{\bar{T}_2} 100 = \frac{534.9 - 288.8}{534.9} 100 = 46.01$$

$$\overline{PR} = \left(1 - \frac{\bar{T}_1}{\bar{T}_2}\right) 100 = \left(1 - \frac{288.8}{534.9}\right) 100 = 46.01$$

here T_2 represents control mean in milligrams, and $\bar{T}_1$ represents the treatment mean in milligrams.

Note that the ANOVA F tests indicate a strong antiperspirant effect and a all sides effect, if any, in this case.

4. Confidence limits about the population mean per cent reduction* are imated as follows:

(a) Using the error mean square (emr) from the original ANOVA (done on logs of the data), compute the standard error of the difference between the treatment and control means

$$SED = \bar{S}_d = \sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}$$

where S_1^2 represents the variance for antiperspirant data; S_2^2 represents the variance for control data; n_1 represents the number of data in the antiperspirant mean; and n_2 represents the number of data in the control mean (under the assumption of homoscedasticity, which was satisfied by the log transformation $S_1^2 = S_2^2$). Because of the experimental design, equal numbers of axillae were used for antiperspirant and control; therefore $n_1 = n_2$. The above expression therefore reduces to

$$\bar{S}_d = \sqrt{\frac{2S^2}{n}}$$

where S^2 is the emr in the ANOVA.
Therefore

$$\bar{S}_d = \sqrt{\frac{2(0.0212)}{12}} = 0.0594$$

*95% confidence limits used in this way are indicators of the precision of the test but *posteriori*, do not imply a probability that the population mean is included in the interval. This interpretation can only be used *a priori*, when one can say that (if $(1-\alpha) = 95$) the population mean will be included in 19 out of 20 identical experiments. *A posteriori*, the mean either is or is not included (12), and a probability estimate is inappropriate.

(b) Compute the difference between the antiperspirant and control means (in terms of logs)

$$\overline{T}_1 = 5.6656: \qquad \overline{T}_2 = 6.2820$$
$$\overline{D} = 5.6656 - 6.2820 = -0.6164$$

note that the difference used is antiperspirant minus control, not the reverse.

(c) Compute the 95% confidence interval about the population difference

$$CL_{0.95} = \overline{D} \pm \bar{S}_d\, t_{0.05}$$

where $\overline{D}$ represents the above difference; $\bar{S}_d$ represents the standard error of the difference; and $t_{0.05}$ represents Student's t at $\alpha = 0.05$.
In the example

$$\overline{D} = -0.6164$$
$$\bar{S}_d = 0.0594$$
$$t_{.05} = 2.228^{\bullet}$$

so that

$$CL_{0.95} = -0.6164 \pm (0.0594)\,(2.228)$$
$$= -0.6164 \pm (0.1323)$$

or

$$-0.4841 \text{ to } -0.7487$$

(d) A difference between the logarithms of two numbers is the ratio of the two numbers when antilogs are taken. Thus

$$\text{antilog } \overline{D} = \frac{\overline{T}_1}{\overline{T}_2}$$

where $\overline{T}_1$ and $\overline{T}_2$ are in milligrams. If we take antilogs of the two confidence limits, we obtain a maximum and minimum ratio of antiperspirant to control milligrams, representing 95% confidence limits about the population mean ratio:

$$\text{antilog } -0.4841 = 0.6163$$
$$\text{antilog } -0.7487 = 0.4730$$
$$PR_1 = (1\text{-}0.6163)\,100 = 38.37$$
$$\overline{PR} \quad \text{(from step 3)} = 46.01$$
$$PR_2 = (1\text{-}0.4730)\,100 = 52.70$$

*At 10 df (the error df in the ANOVA).

B. *RM Calculations (Without Transformations)*

The method to be illustrated manipulates individual adjusted ratios and is commonly used. Another, not illustrated here, uses individual per cent reduction values computed from single adjusted ratios as the basic statistics. Both methods are incorrect due to the fact that they use statistics (arithmetic means, estimates of standard deviations, and standard errors), which yield biased results because of the nonnormal character of the ratios and per cent reduction values. An additional source of bias in the error estimates is lack of provision for the influence of residual sides effects, since it is unlikely, as discussed above, that the adjustment procedure removes them completely. Procedure B is illustrated, therefore, solely to show the departure from the correct values given by method A. For simplicity, in our examples of the RM we use mean pretest ratios derived from two successive days of pad B runs, and a single hot-room test using pad B only is illustrated.

This procedure is commonly done as follows:

1. Individual pretest and posttest ratios are computed from the milligram data. For the data of the example, these were given in Table I.

2. Arithmetic means of the pretest ratios are calculated, and the adjusted ratios are computed by dividing the posttest ratios by these mean pretest ratios. For example, for subject 19 in Table I (chosen because of the fairly large disagreement between the two pretest ratios)

$$\begin{aligned}
&\text{Pretest ratio, day 1} = R_{p1} = 0.421\\
&\text{Pretest ratio, day 2} = R_{p2} = 0.763\\
&\text{Mean pretest ratio} = R_p = 0.592\\
&\text{Posttest ratio} = R_t = 0.429\\
&\text{Adjusted ratio} = \overline{R'} = R_t/R_p = 0.725
\end{aligned}$$

In a similar way, $\overline{R'}$ values for each of the 12 subjects of Table I are computed. These are shown in Table IV (last column).

The overall mean posttest ratio $\overline{R'}$ is obtained by averaging the adjusted ratios R′ in Table IV ($\overline{R'} = 0.6173$).

The variance, standard deviation, and standard error of the mean are computed from the adjusted ratios $\overline{R'}$

$$\begin{aligned}
&\text{Estimated variance} = S^2 = 0.0235\\
&\text{Estimated standard deviation} = S_{x1} = 0.1533\\
&\text{Estimated standard error} = S_{\bar{x}} = 0.0442
\end{aligned}$$

95% confidence limits about $\overline{R'}$ are computed using $S_{\bar{x}}$ and Student's t at $p = 0.05$ and 11 degrees of freedom

$$\begin{aligned}
CL_{95} &= \overline{R'} \pm S_{\bar{x}}\, t_{0.05}\\
&= 0.6173 \pm (0.0442)\,(2.201)\\
&= 0.7146 \text{ to } 0.5200
\end{aligned}$$

Table IV
Summary of Ratios for Method B

Subject Number	R_{p_1}	R_{p_2}	$\overline{R_p}$	R_t
13	1.012	0.980	0.996	0.417
14	1.084	1.117	1.101	0.664
15	1.135	1.089	1.112	0.800
16	1.359	1.339	1.349	0.517
17	0.978	0.896	0.937	0.676
18	0.600	0.709	0.655	0.517
19	0.421	0.763	0.592	0.429
20	0.830	0.757	0.794	0.643
21	0.837	0.829	0.833	0.567
22	0.820	0.779	0.800	0.465
23	1.043	1.154	1.099	0.412
24	0.746	0.956	0.851	0.510

The upper and lower confidence limits about the mean per cent reduc (PR_1, PR_2) are computed from these, and the mean per cent reduction $\overline{P}$ computed from $\overline{R'}$

$$\text{UCL for } \overline{R'} = 0.7146$$
$$\text{mean ratio, } \overline{R'} = 0.6173$$
$$\text{LCL for } \overline{R'} = 0.5200$$
$$PR_1 = (1\text{-}0.5200)100 = 48.00$$
$$\overline{PR} = (1\text{-}0.6173)100 = 38.27$$
$$PR_2 = (1\text{-}0.7146)100 = 28.54$$

C. *RM Calculations With Transformation*

This method takes into account the fact that correct estimates of the n ratios and other statistics cannot be obtained by method B because neithe ratios nor the milligram values from which they are derived are normally tributed. In addition, despite the adjustment procedure, some sides eff may remain in the data, and therefore the error estimate must be obta from the data after accounting for these. Since method C also uses adju ratios, however, it cannot compensate for any distortion in the per cent re tion estimates, which may be introduced by the adjustment procedure. Th fore, although the method is more nearly correct than B, it still may not correct results. Note also that, quite aside from these considerations, the e estimate yielded by method C has a different composition than that of met A, although it is not necessarily incorrect.

The normality problem is handled by transforming the ratios to their l rithms before manipulating them, then back-transforming them. Since

Table V
Summary of Ratios for Method C

Subject Number	Treatment on	R_{p_1}	R_{p_2}	$\bar{R}_G$	R_t	R'	R'_L
13	R	1.012	0.980	0.996	0.417	0.419	−0.870
14	R	1.084	1.117	1.100	0.664	0.604	−0.504
15	R	1.135	1.089	1.112	0.800	0.719	−0.330
16	R	1.359	1.339	1.349	0.517	0.383	−0.960
17	R	0.978	0.896	0.936	0.676	0.722	−0.326
18	R	0.600	0.709	0.652	0.517	0.793	−0.232
19	L	0.421	0.763	0.567	0.429	0.757	−0.278
20	L	0.830	0.757	0.793	0.643	0.811	−0.209
21	L	0.837	0.829	0.833	0.567	0.681	−0.384
22	L	0.820	0.779	0.799	0.465	0.582	−0.541
23	L	1.043	1.154	1.097	0.412	0.376	−0.978
24	L	0.746	0.956	0.844	0.510	0.604	−0.504

original milligram data are log normally distributed, it can be shown that their ratios, as well as the adjusted ratios (which are ratios of ratios) are also log normal. Interestingly, if the milligram data had been normal, the resulting ratios would not have been log normal, and a much more complex transformation would then have been required.

Referring to Table I, and again using the data from subject 19, we get the following:

1. For each subject, obtain the geometric mean of the pretest ratios

$$\begin{aligned}
&\text{Day 1 pretest ratio} && = 0.421; && \ln 0.421 && = -0.865\\
&\text{Day 2 pretest ratio} && = 0.763; && \ln 0.763 && = -0.270\\
&\text{Arithmetic mean of logs} && = (-0.865 - 0.270)/2 && && = -0.568\\
&\text{Antilog of mean} && = \text{geometric mean} && = && 0.567
\end{aligned}$$

(The close agreement between the mean of the logs and its antilog is a result of the particular value of the mean.)

2. Adjust the posttest ratios in the ordinary way, using these geometric mean pretest ratios

$$\bar{R}' = \frac{0.429}{0.567} = 0.757$$

3. Take natural logarithms of all adjusted posttest ratios. Table V lists these for the example data of Table I. In Table V, R_{p1}, and R_{p2} are the pretest ratios; $\bar{R}_G$ is the geometric means of the pretest ratios for each subject; R_t is the posttest ratio; R' is the adjusted ratio; and R'_L is the natural logarithm of R'. Note the differences between the R' values in Table IV and those in Table V. These are due to the different method of computing the mean pretest ratio.

Table VI
ANOVA of R'_L Data of Table V
(Method C)

Source of Variation	DF	SS	MS	F
Due to side treated	1	0.0090	0.0090	0.106
Error	10	0.8470	0.0847	
Total	11	0.8560		

Most are very similar, but as can be seen, the difference increases as a function of the disagreement between the two pretest ratios. In experiments in which more than two pretest ratios are used, or with larger sets of subjects, such substantial disagreements will be more numerous.

4. Using the log-transformed adjusted ratios R'_L, carry out the ANOVA of Table VI (separating "side treated" and error). This procedure is equivalent to doing a t-test for side treated (the square root of F is t in this case), but is done as a convenient method for obtaining an error estimate free of any sides effect.

5. The standard error of the mean of the logs of the adjusted ratios is obtained from the ems in Table VI. Confidence limit are then calculated about the mean value of R_L, antilogs taken, and the per cent reduction and its 95% confidence limits obtained.

$$\text{Mean of logs of adjusted ratios} = \overline{R'_L} = -0.5097$$

$$\text{Standard error of mean} = \sqrt{\frac{0.0847}{12}} = 0.0840$$

$$t_{0.05} \text{ at } 10 \text{ df} = 2.228$$

$$CL_{0.95} = \overline{R'_L} \pm S_{\bar{x}}\, t_{0.05}$$
$$= -0.5097 \pm (0.0840)\,(2.228)$$
$$= -0.3226 \text{ to } -0.6969$$

antilog (−0.6969) = 0.498 (lower 95% CL for ratio)
antilog (−0.5097) = 0.601 (mean ratio)
antilog (−0.3226) = 0.724 (upper 95% CL for ratio)

$PR_1 = (1\text{-}0.724)100 = 27.60$ (lower 95% CL for PR)
$\overline{PR} = (1\text{-}0.601)100 = 39.90$ (mean per cent reduction)
$PR_2 = (1\text{-}0.498)100 = 50.20$ (upper 95% CL for PR)

Summary

Methods A, B, and C were applied to 5 sets of two-sample antiperspirant data involving varying numbers of subjects, following the procedures just illustrated. The results are summarized in Table VII.

Table VII
Comparison of Results of Analysis of Two-Sample Data Sets Using Methods A, B, and C

Data Set Number	Statistic	Method A (SSEM)	Method B (RM)	Method C (RM)
1. (used in examples)	Number of subjects	12	12	12
	Mean PR	46.01	38.27	39.90
	CL_{95} about PR	38.4 to 52.7	28.5 to 48.0	**27.6** to 50.2
	Width of CL_{95}	14.3	19.5	22.6
2.	Number of subjects	36	36	36
	Mean PR	20.51	15.03	16.61
	CL_{95} about PR	13.6 to 26.8	9.3 to 20.8	11.0 to 21.9
	Width of CL_{95}	13.2	11.5	10.9
3.[a]	Number of subjects	36	36	**36**
	Mean PR	44.14	39.91	43.10
	CL_{95} about PR	36.1 to 51.2	33.6 to 46.3	35.7 to 49.6
	Width of CL_{95}	15.1	12.7	13.9
4.	Number of subjects	8	8	8
	Mean PR	19.61	21.60	24.56
	CL_{95} about PR	−11.7 to 42.1	1.6 to 41.6	3.7 to 40.9
	Width of CL_{95}	53.8	40.0	37.2
5.	Number of subjects	12	12	12
	Mean PR	14.07	14.19	15.45
	CL_{95} about PR	1.3 to 25.2	3.2 to 25.2	**3.8** to 25.7
	Width of CL_{95}	23.9	28.4	21.9

[a]This set showed a moderate sides effect in methods A and C. The sides effects in the other sets, before correction with ratios, appeared to be quite small.

Miscellaneous Related Topics

Sympathetic Effect

An hypothesis, which has been advanced occassionally, suggests that an effective antiperspirant applied to one axilla might stimulate additional sweating in the opposite (control) axilla. An argument in favor of the use of the RM, in spite of the disadvantages suggested in the foregoing discussion, might be the assumption that it will correct for this "cork effect" because it uses pretest control values, which are obviously unaffected by the antiperspirant treatment. We made calculations from the 5 sets of data summarized in Table VII, using the SSEM method, but with pretreatment instead of concurrent controls. Of course, this procedure introduces substantial additional variation,

but with these data we have found no evidence for the effect. If it existed, the expectation would be that the point estimates of per cent reduction obtained with the regular SSEM method (concurrent controls) would be biased in an upward direction (inflated), while the use of pretest controls should give lower PR values. If the cork effect were small or nonexistent, on the other hand, the two methods should vary in their outcomes in a random manner with, of course, larger errors when *a priori* controls are used. Our results on these sets of data do not show any consistency which could be considered significant; there were 4 cases in which the *a priori* controls yielded higher per cent reductions than those obtained with concurrent controls and one in which they were lower. We therefore believe that any cork effect in the data—if it exists at all—must be small.

Other Models

The discussion and examples in this paper have been confined to experiments in which only two "treatments" were considered: antiperspirant and control. However, it is possible to use three or even a greater number of treatments. In fact, at this laboratory the usual evaluation is a three-sample procedure in which a control and two different antiperspirants are tested. The ordinary SSEM procedure is used, but the design, instead of being a crossover, is a form of balanced incomplete blocks structure known as a Youden square. A schematic picture of such a design is shown in Fig. 2. The analysis is substantially more complex than that of the crossover, but is easily and quickly done with even small computer facilities and can be done in a few hours with a desk calculator. A similar design can be used for 5 samples. This is illustrated in Fig. 3.

Finally, there is a model for the two-sample test which may be superior in some ways to the crossover we have recommended. We are just beginning to

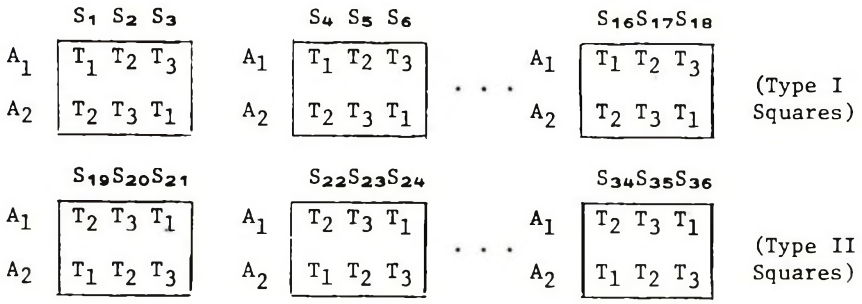

Figure 2. Three-sample test design (2 x 3 Youden squares) (illustrated for 36 subjects): A_i represents axillae, where i equals 1 or 2; T_j represents treatments (including control), where j = 1, 2, or 3; and S_k represents subjects, where k = 1, 2 . . . 36. (Appropriate randomization not shown.)

(Type I Squares)

	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}		S_{11}	S_{12}	S_{13}	S_{14}	S_{15}	S_{16}	S_{17}	S_{18}	S_{19}	S_{20}
A_1	T_1	T_2	T_3	T_4	T_5	T_1	T_2	T_3	T_4	T_5	A_1	T_1	T_2	T_3	T_4	T_5	T_1	T_2	T_3	T_4	T_5
A_2	T_2	T_5	T_4	T_1	T_3	T_3	T_4	T_2	T_5	T_1	A_2	T_2	T_5	T_4	T_1	T_3	T_3	T_4	T_2	T_5	T_1

(Type II Squares)

	S_{21}	S_{22}	S_{23}	S_{24}	S_{25}	S_{26}	S_{27}	S_{28}	S_{29}	S_{30}		S_{31}	S_{32}	S_{33}	S_{34}	S_{35}	S_{36}	S_{37}	S_{38}	S_{39}	S_{40}
A_1	T_2	T_5	T_4	T_1	T_3	T_3	T_4	T_2	T_5	T_1	A_1	T_2	T_5	T_4	T_1	T_3	T_3	T_4	T_2	T_5	T_1
A_2	T_1	T_2	T_3	T_4	T_5	T_1	T_2	T_3	T_4	T_5	A_2	T_1	T_2	T_3	T_4	T_5	T_1	T_2	T_3	T_4	T_5

Figure 3. Five-sample test design (2 x 10 Youden squares) (illustrated for 40 Subjects). A_i represents axillae, where i equals 1 or 2; T_j represents treatments (including control), where $j = 1, 2, \ldots 5$; and S_k represents subjects, where $k = 1, 2 \ldots 40$. (Appropriate randomization not shown.)

experiment with it, although it represents a well-known statistical design. It can be characterized as a 2^2 factorial design in which one degree of freedom is confounded with pairs of subjects. The laboratory protocol (it is a two-sample design) would be almost the same as the regular crossover of Fig. 1, but the error structure is more explicit and it will be possible to use it not only for the usual evaluations but also as a means of detecting treatments x sides interactions.

Conclusions and Discussion

General

In evaluating either the SSEM or the RM, the central questions are these: (1) does the method give an undistorted estimate of the true (population) per cent reduction; (2) if so, is it satisfactorily precise; and (3) how easy is it to use?

The SSEM analysis removes the average sides and subjects effects from error. The width of the confidence limits it produces is a function of the number of subjects used in the experiment, as well as the basic error associated with the protocol. The per cent reduction estimate can be shown to be unbiased, and the precision of the estimate is as good as can be obtained with a given number of subjects, using the crossover model.

The RM gives estimates of per cent reduction which have been modified by the *a priori* mean ratio obtained at the time of the pretest measurements. If this mean ratio were constant, it would not alter the per cent reduction obtained relative to that of the SSEM. To the extent that this is not true, the per cent reduction will be different than that given by the SSEM. Since it has been shown that the ratios can be quite variable, it follows that the per cent reduction estimates obtained may be substantially altered. This difference in the estimates is obviously a function of the degree of variability of the pretest ratios. Under these circumstances, the precision of the estimate becomes irrelevant even though it is a function of the number of pretest determinations as well as the factors operating in the SSEM. A questionable estimate is not improved by reducing its variability.

It is possible, however, to utilize pretest data in a statistically valid manner to adjust posttest results by the use of an analysis of covariance, and this procedure might be shown to give more accurate, and precise estimates of per cent reduction than those given by the SSEM, if the pretest data are sufficiently well-correlated with the posttest ratio. The mathematics of such an analysis, however, differ from those of the ratio adjustment procedure, and it is impossible to predict results without experimentation. When time permits, we plan to investigate this matter. Meanwhile we recommend the SSEM over the RM as the scientifically valid method, as well as the one of choice on the bases of simplicity and low cost.

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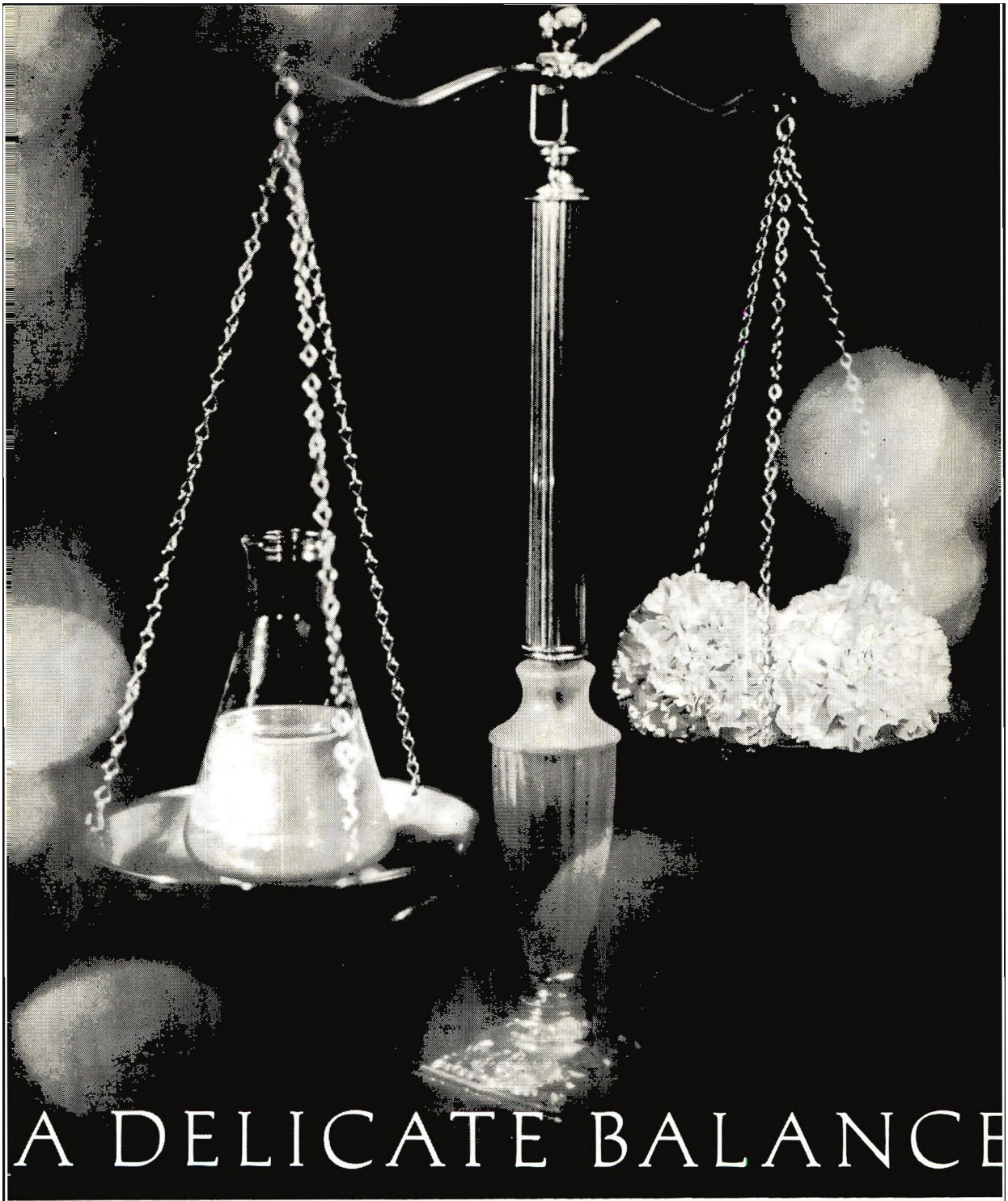

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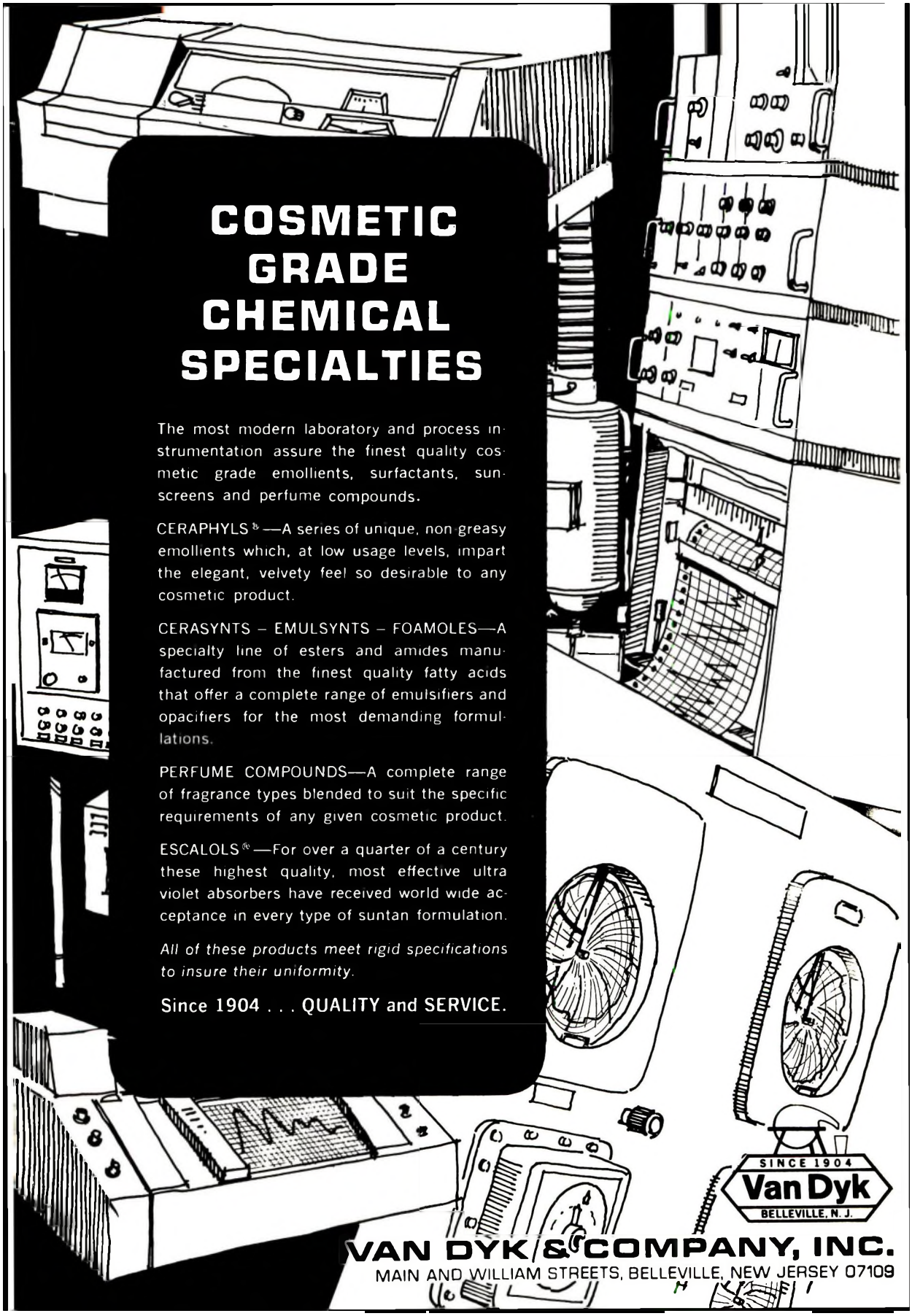

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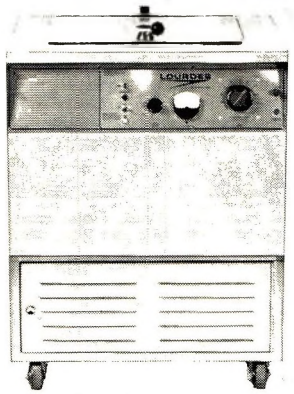

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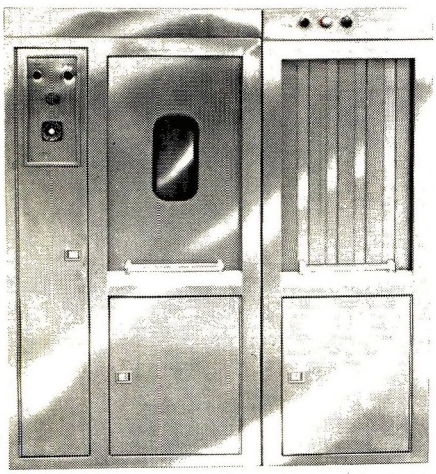

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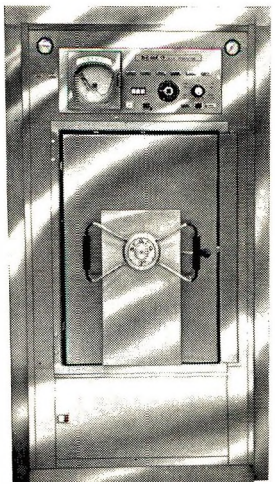

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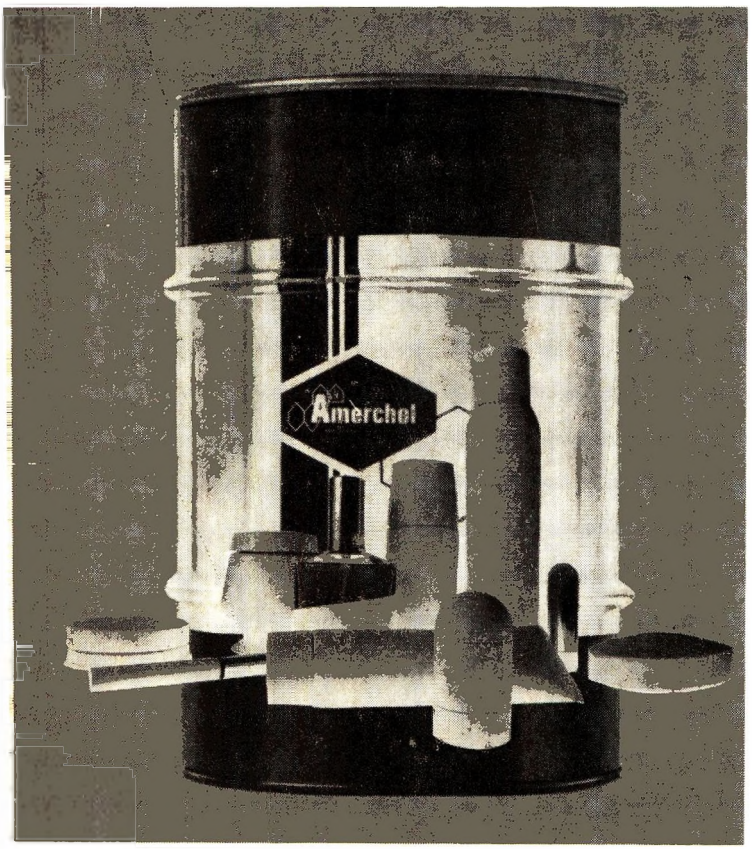

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