Chromatography on Silicic Acid of the Unsaponifiable Matter of Fats[1]

P. CAPELLA, G. de ZOTTI, G. S. RICCA, A. F. VALENTINI, and G. JACINI,
Stazione Sperimentale per le Industrie degli Olii e dei Grassi, Milan, Italy

A method has been described for the separation of unsaponifiables into their major chemical classes by silicic acid adsorption chromatography. Methods are also presented for the isolation of unsaponifiables free of fatty acids. The chromatographic procedure was tested on synthetic mixtures of hydrocarbons, esters, alcohols, and sterols and then applied to the unsaponifiables of extracted and pressed olive oils, soybean, teaseed, and rapeseed oils, lard, and tallow. The major sterol of all the unsaponifiables examined was found to be β-sitosterol. Analytical data such as infrared analysis, carbon-hydrogen analysis, melting points of derivatives, and paper chromatography of the sterol fractions are also presented.

The unsaponifiable part of fatty materials has always held the attention of researchers since it contains substances of particular scientific and practical interest which are related in some manner to the origin of the oil. Although some information is available on the composition of the unsaponifiables of oils, most of the data are concerned only with such physical and chemical properties as the melting and solidification points (4,8,22), the iodine value (1,15), and the refractive index (21). Since these values reflect little of the complex nature of the material, we thought it would be of interest to develop a general method for the systematic resolution of unsaponifiables. Previous publications of a similar nature were the work of Ruzicka and coworkers (24) on the unsaponifiables of extracts of swine organs, of Fuhrmann (14) on the unsaponifiables of olive oils from Tunisia, and of Cruz-Auñon (5) on the unsaponifiables of soybean and cottonseed oil.

This paper describes the isolation of unsaponifiables from fats and oils and the separation of this material into various components by elution chromatography on silicic acid columns. The elution is performed by a series of solvents or mixtures of solvents of increasing polarity and permits the quantitative isolation of the following series of components: The saturated hydrocarbons; the polyene hydrocarbons (squalene); the waxes; the esters of sterols; the aliphatic alcohols; a group of materials the nature of which will make up a separate paper, containing triterpene alcohols; the free sterols; and a group of substances still unidentified which may be alteration products.

Although the method was developed to study the unsaponifiables of extracted olive oil, it may also be applied to other fats and oils. The resolution of unsaponifiables containing compounds of a different chemical type from those listed will, of course, depend upon the functional group of the compounds, and application of the elution scheme will, in general, permit their separation. As an example of this, in the separation of teaseed oil unsaponifiables (Figure 7), we have isolated by our method a group of substances giving the so-called Fitelson reaction (11).

Methods

Preparation of Unsaponifiables. Fifty grams of oil are saponified by boiling on a water bath with 500 ml. of 2-N alcoholic potassium hydroxide for 1 hr., then transferred to a 5-liter separatory funnel containing 500 ml. of distilled water. The flask is rinsed with 500 ml. of water, which are also added to the funnel; 500 ml. of peroxide-free ethyl ether of recent distillation are added, and the mixture is vigorously shaken. After standing for 12 hrs., the aqueous layer is removed and re-extracted twice with 1 liter of ethyl ether, allowing only 15 min. for the layers to separate in these extractions. The ethereal extracts are combined and washed to neutrality, using about 250 ml. of water for each wash.

After the first water wash a 2-hr. standing period is required for complete separation of the layers, but the succeeding washes require only 10 min. The ether solution is dried over anhydrous sodium sulfate, and the solvent is evaporated under reduced pressure in a stream of nitrogen.

Removal of Fatty Acids from Unsaponifiables. The unsaponifiables thus obtained always contain considerable amounts of free fatty acids which must be removed. One of the two following methods is preferred for their removal rather than washing with alkaline solutions.

About 400 mg. of unsaponifiable matter dissolved in smallest possible amount of chloroform are added to a column containing 10 g. of basic copper carbonate [$CuCO_3 \cdot 2\ Cu\ (OH)_2$], which had previously been washed with 100 ml. of $CHCl_3$. The flow rate of the column can be increased by prior mixing of the copper carbonate with Celite in a one-to-three ratio. The unsaponifiables are eluted with 450 ml. of chloroform, and the final 10 ml. of this should leave a residue of less than 1 mg.

An alternate procedure for fatty acid removal employs a column of 24 g. of Brockman alumina. The sample is added to the column in a small volume of ethyl ether, and the unsaponifiables are removed with 250 ml. of the same solvent.

Chromatography of Unsaponifiables. Approximately 100 mg. of unsaponifiable matter dissolved in a minimum volume of hexane was added to the top of a 13-mm. chromatographic column containing 3 g. (30-to-1 ratio adsorbent to sample) of Mallinckrodt's 100-mesh silicic acid. After the charge had almost completely passed into the column, the sides were washed twice with 1–2 ml. of hexane, and elution with the first solvent was started. Since extreme care must be taken to prevent the column from becoming dry when changing from one solvent to another, a stopcock or other means of discontinuing the column flow should

---

[1] Report submitted to the Fourth Congress of the International Society for the Study of Fatty Materials, Graz, September 1–3, 1959.

be provided at the bottom outlet of the column. Fractions of 4 ml. each were collected in small weighed flasks, and when successive fractions contained no residue, the next solvent of increasing polarity was added according to the scheme outlined in Table I. Changes in the eluting solvent were accomplished by removing the solvent remaining above the silicic acid with a pipette while the column flow was stopped. The next solvent was added slowly to prevent disruption of the column structure.

Analysis. The weight of each fraction was determined by evaporating the solvent in a small weighed flask in a stream of nitrogen and weighing the residue. The analytical data were obtained on the various fractions by semimicro methods. The presence of sterols was detected by the well-known Liebermann-Burchard reaction (3); esters were revealed by the formation of the corresponding ferric hydroxamate, according to Goddu *et al.* (17). The infrared spectra were recorded by a Baird Model 4-55 double beam spectrophotometer.

Results and Discussion

Although silicic-acid chromatographic adsorption techniques have been widely used for the separation of fatty materials into their major components according to their polarity, the further separation of the classes by this procedure into individual compounds is much more difficult and requires very special conditions. Likewise, in our case, the unsaponifiables were resolved into eight fractions corresponding to as many series, each of which consisted of various homologous substances. It was possible to extend the separation of the classes by paper chromatography, particularly Fractions E and G (Table I), the higher alcohols (31), and the sterols (7), respectively.

TABLE I

Elution Scheme, Chromatographic Separation of Unsaponifiables

Fraction	Eluant	Eluted substances
A	Hexane	Paraffins and olefins
B	Carbon disulfide	Polyene hydrocarbons
C	Hexane 5% Benzene	Waxes
D	Hexane 10% benzene	Sterol esters
	Hexane 20% benzene	Sterol esters
E	Hexane 30% benzene	Higher aliphatic alcohols
F	Hexane 2% ethyl ether	Triterpenoid alcohols
G	Hexane 8% ethyl ether	Sterols
H	Chloroform 5% methanol	Unidentified substances

The results of the present work pertain only to unsaponifiables as they are usually defined. The material present in the unsaponifiables and isolated by chromatography was produced by saponification. Thus the waxes and sterol esters were replaced by free alcohols and sterols. Working on model mixtures containing squalene, we have found little evidence of structural changes produced by the saponification, extraction, and adsorption treatment although there is the possibility of structural modifications such as the shifting of double bonds.

Preparation of Unsaponifiables. In our experience, unsaponifiables produced by standard methods contained high percentages of free fatty acids. These free acids later contaminated the triterpene alcohol and free sterol fractions and were difficult to remove. By working with model mixtures of free acids, β-sitosterol and docosanol, we found that passage of the mixture over basic copper carbonate effectively removed the free acids and permitted quantitative recovery of the unsaponifiables which are not absorbed. The recovery of the fatty acids was accomplished by suspending the copper carbonate in ether and treating with dilute HCl.

By measuring the peroxide value before and after the chromatographic treatment, it was also shown that the treatment induced no further oxidation of the material.

Several treatments based on neutralization of the fatty acids, followed by extraction of the unsaponifiables, were tried. The best of these, although of greater complexity than the adsorption procedure, was to neutralize the mixture with stoichiometric amounts of lithium ethylate in alcohol solution. This was then extracted three times with hot benzene to remove the unsaponifiables. It was possible by this procedure to produce unsaponifiables of zero acid number and with no contamination of metallic lithium.

TABLE II

Separation of Synthetic Mixture[a]

Fraction	Eluant	Eluted substance	Wt. recovered (mg.)
Tube No.			
1– 6 A	Carbon disulfide	....	......
7– 47 B	Hexane 5% benzene	Acetyldocosanol	25.5
47– 76 C	Hexane 10% benzene	Acetyl-β-sitosterol	25.0
77– 86 D	Hexane 20% Benzene	....	......
87–110 E	Hexane 30% benzene	Docosanol	25.5
111–129 F	Hexane 8% ethyl-ether	β-Sitosterol	24.5

[a] Synthetic mixture composed of 25.1 mg. of acetyldocosanol, 25.6 mg. of acetyl-β-sitosterol, 25.1 mg. of docosanol, and 25.0 mg. of β-sitosterol.

Chromatography. We found little in the literature directly related to the application of chromatography to the unsaponifiables. The investigations by Trappe (29,30), by Fillerup and Mead (10), by Borgstrom (2), and by Spengler (25,26,27,28), and recently by Hirsch (20) are more properly concerned with lipids. In the development of our procedure, model mixtures of the type of compounds found in unsaponifiables were subjected to various chromatographic schemes. Although the separation of hydrocarbons from unsaponifiables had been reported (9,12,13,18,19,20,23), it is interesting to note that we were able to separate paraffins and monoene-hydrocarbons from the polyene squalene. The former were first eluted with hexane, and the latter then removed with carbon disulfide.

Table II gives the results obtained with a model mixture of acetyl-docosanol, acetyl-β-sitosterol, docosanol, and β-sitosterol, every component of which was easily prepared in a pure form and the chromatographic behavior of which is closely similar to that of natural mixtures of waxes, sterol esters, fatty alcohols, and sterols. This mixture was chromatographed according to the elution scheme shown in Table II that differs only slightly from that of Table I. Figure 1 depicts graphically the trend of this separation.

When the scheme of Table II was applied to the separation of the unsaponifiables of olive oil, it was observed that the sterol fraction was contaminated by a compound giving a yellow Liebermann-Burchard test. The elution scheme was modified to include the use of 2% ethyl ether in hexane prior to using 8% ethyl ether (Table I), resulting in complete separation of material from the sterol fraction. While the data indicate that this material contains a triterpene alcohol, a separate investigation is in progress to characterize it.

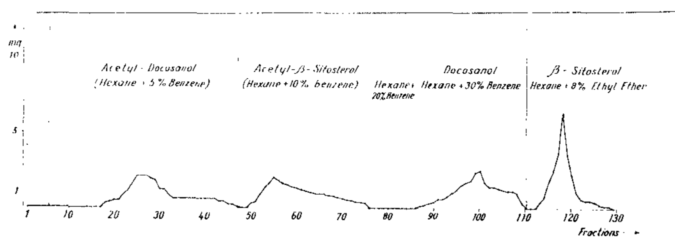

FIG. 1. Elution of synthetic mixture.

From the many preliminary tests conducted, the final elution scheme given in Table I was adopted and was found capable of separating the classes of compounds found in the unsaponifiables.

Identification of Chromatographic Fractions. The unsaponifiables of extracted olive oil were resolved by our method into eight distinct fractions according to the scheme of Table I. The graphic representation of this separation is shown in Figure 2. The characteristics of each fraction are reported below:

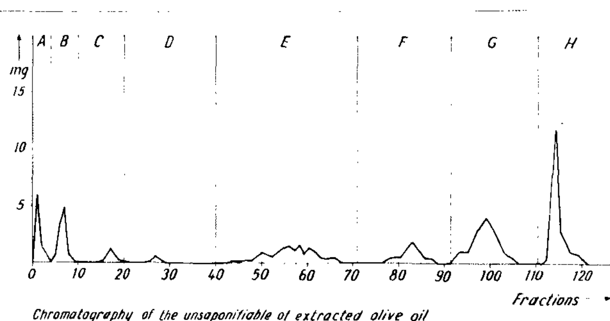

FIG. 2. Elution of extracted olive oil unsaponifiable.

Fraction A. This was a white, waxy solid after crystallization from acetone and had a melting point of 62.5°C. Carbon-hydrogen values (C = 84.97%, H = 14.93%) agree well with those calculated for a $C_{30}H_{62}$ hydrocarbon. Infrared analysis revealed only CH_2 and CH_3 groups.

Fraction B. The fraction eluted with carbon disulfide was a colorless liquid with a blue fluorescence. This fluorescence was probably due to contamination as it disappeared when the fraction was rechromatographed. The index of refraction of this and similar materials was 1.4961 to 1.4964 in the range of reliable values reported for synthetic squalene. The infrared curve shown in Figure 3 is identical with those for synthetic and natural squalene.

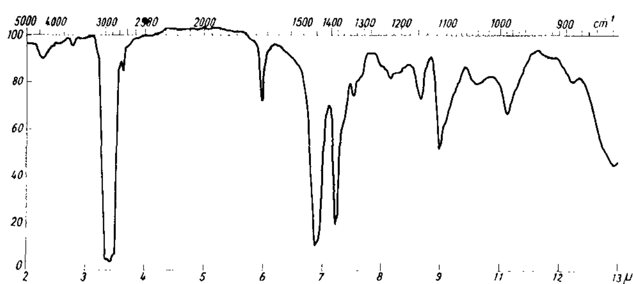

FIG. 3. Infrared spectrum of squalene isolated from olive oil unsaponifiable.

Fraction C. The small amount of material contained in this fraction allowed only a general identification. The presence of ester groups was confirmed by both infrared analysis and chemical test (ferric hydroxamate formation).

Fraction D. This was also a very small fraction and did not permit a thorough examination. It gave a positive sterol test and is very probably sterol esters. This material and Fraction C must be products of incomplete saponification of original oil.

Fraction E. This material was crystallized from 96% ethanol and yielded a waxy product melting at 78.5°C. The carbon-hydrogen values (C = 81.7%, H = 14.02%) are very similar to those calculated for the higher alcohol $C_{26}H_{54}O$ (C = 81.60%, H = 14.21%). The presence of higher alcohols in the unsaponifiables has been reported by several investigators, including Fuhrmann (14), Gracian (16), and others.

The acetyl derivative after crystallization melted at 60°C., and the carbon-hydrogen values (C = 79.05%, H = 12.89%) were similar to those calculated for a $C_{28}H_{56}O_2$ ester. (C = 79.24%, H = 13.20%). The infrared spectrum confirmed the materials as fatty alcohols. The material was subjected to paper chromatography, and this research will be the subject of a special paper (31).

Fraction F. This is a very complex material, and its presence necessitated the modification of our original elution scheme as previously mentioned in the text. The data so far in our possession would lead us to assume that it is a group of substances common to many fats. The fraction contains two groups of materials; one of these forms is an insoluble digitonide although it is not a sterol; the other does not form a complex with digitonin. A separate work is in progress concerning the composition of Peak F.

Fraction G. This was chromatographed on paper using the same techniques as described in previous publications (6,7). β-Sitosterol appeared to be the main sterol present, but in the latter fractions there were at least three other sterols present, one of which gave an R_f value very close to that of cholesterol.

The early fractions (Figure 2), 90–97, were combined and crystallized from absolute alcohol and yielded a product melting at 134.5–135.0°C. When this was acetylated and recrystallized from alcohol it melted at 119.0–119.5°C. A mixed melting point with acetyl-β-sitosterol of melting point 124.0°C. gave a melting point of 119.0–119.5°C. The carbon-hydrogen analysis (C = 81.77%, H = 11.36%) agreed with that calculated for $C_{29}H_{48}O$ (C = 81.52%, H = 11.47%).

The acetyl derivative was brominated and the carbon-hydrogen analysis (C = 60.77%, H = 8.11%) was very close to that for $C_{29}H_{48}Br_2O$.

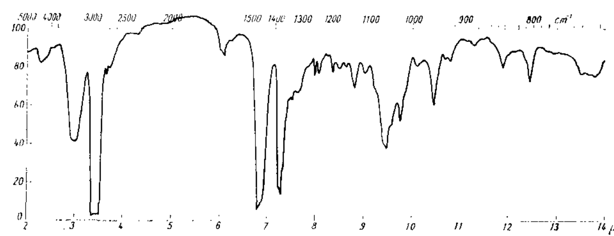

FIG. 4. Infrared spectrum of β-sitosterol isolated from olive oil unsaponifiable.

Figure 4 presents the infrared spectrum of the sterol in Nujol suspension, and it is characteristic of a sterol containing unsaturation in the 5–6 position. It is also identical with the spectrum of β-sitosterol isolated from tall oil. Despite the low melting point of the acetyl derivative, it seems to us that the main olive oil sterol is β-sitosterol, associated in minor amounts with at least three other sterol-like materials.

Unsaponifiables of Other Oils. The method was also applied to the unsaponifiables of pressed olive oil, soybean oil, teaseed oil, rapeseed oil, lard, and tallow. The curves of these separations are shown respectively in Figures 5 to 10. It is interesting to note that the material of Fraction F, occurring just prior to the sterols, contains the substance that gives the yellow Liebermann-Burchard test. Paper chromatograms of all the sterol fractions confirmed the presence of only one main sterol component and several minor sterol components in each. The nature of these minor sterols will be the subject of a future work.

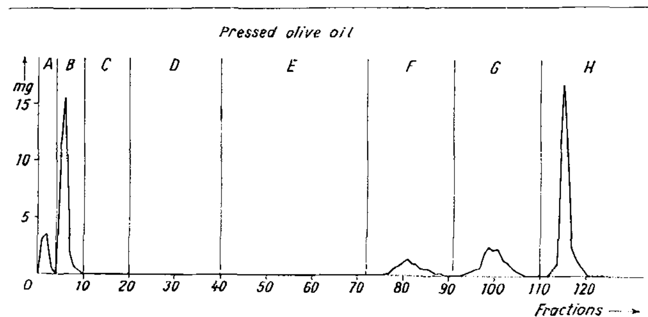

Fig. 5. Elution of pressed olive oil unsaponifiable.

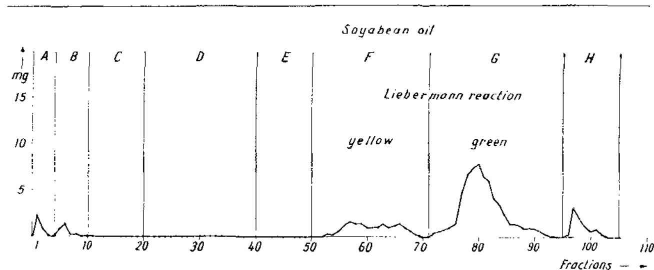

Fig. 6. Elution of soybean oil unsaponifiable.

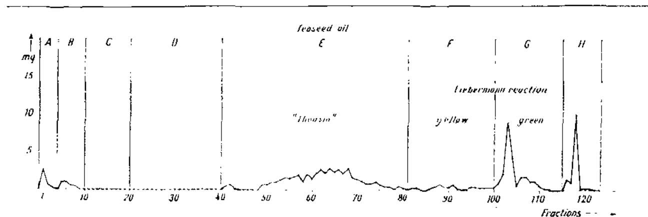

Fig. 7. Elution of teaseed oil unsaponifiable

Acknowledgment

The authors wish to acknowledge the kind editorial assistance of F. E. Luddy of the Eastern Regional Research Laboratory.

REFERENCES

1. Bolton, E. R., and Williams, K. A., Analyst, *55*, 5 (1930).
2. Borgstrom, B., Acta Physiol. Scand., *25*, 101 (1952).
3. Burchard, H., Chem. Zentr., *25*, 1 (1890).
4. Carola, C., Olii Min. Grassi Sap. Colori Vern., *26*, 5 (1949).
5. Cruz-Auñon, F., Bol. Inst. Nacl. Invest. Agron. (Madrid), *15* (32) 187 (1955).
6. deZotti, G., and Capella, P., La Chimica e l'Industria, *40*, 651 (1958).
7. deZotti, G., Capella, P., and Jacini, G., Fette und Seifen, *61*, 1114 (1959).
8. Dorta, G., Olii Min. Grassi Sap Colori Vern., *18*, 5 (1938).
9. Drummond, J. C., Analyst, *60*, 23 (1935).
10. Fillerup, D. L., and Mead, J. F., Proc. Soc. Exp. Biol. and Med., *83*, 574 (1953).
11. Fitelson, J., J. Assoc. Off. Agr. Chem., *19*, 493 (1936).
12. *Ibid.*, *26*, 506 (1943).
13. *Ibid.*, *28*, 282 (1945).
14. Fuhrmann, R., J. de Recherches C.N.R.S., *325* (1954).
15. Gracian, J., and Vioque, E., Grasas y Aceites, *4*, 58 (1953).
16. Gracian, J., and Martel, J., Grasas y Aceites, *8*, 3 (1957).
17. Goddu, R. F., Le Blanc, N. F., and Wright, C. M., Anal. Chem. *27*, 1251 (1955).
18. Hadorn, H., and Jungkunz, R., Mitt Gebl Lebensm. Hyg. *40*, 61 (1949).
19. Hadorn, H., and Jungkunz, R., Mitt Gebl Lebensm. Hyg., *41*, 435 (1950).
20. Hirsh, J. M., and Ahrens, E. H. Jr., J. Biol. Chem. *233*, 311 (1958).

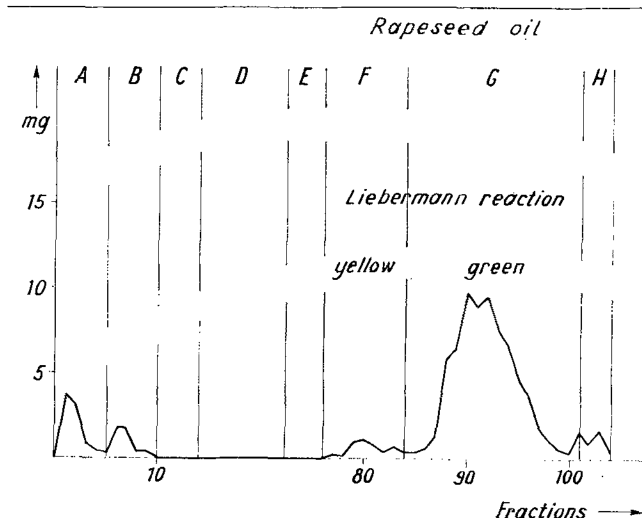

Fig. 8. Elution of rapeseed oil unsaponifiable.

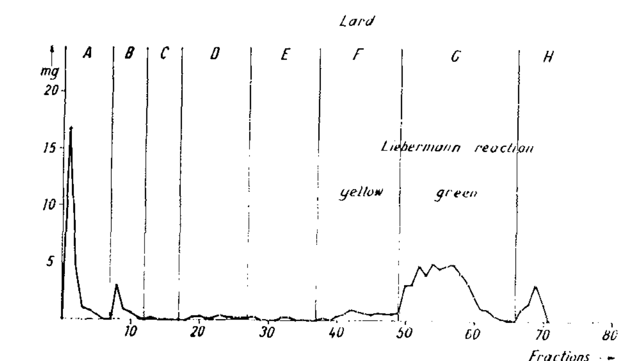

Fig. 9. Elution of lard unsaponifiable.

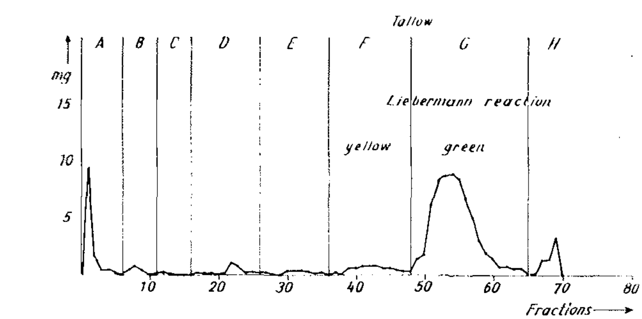

Fig. 10. Elution of tallow unsaponifiable.

21. Kofler, L., and Opfer-Schaum, R., Fette und Seifen, *48*, 49 (1941).
22. Loew, G., Ind. y Quimica, *9*, 9 (1947).
23. Marcellet, H., Comptes Rendu., *202*, 867 (1936).
24. Prelog, V., Ruzicka, L., and Stern, P., Helv. Chim. Acta., *26*, 2222 (1943).
25. Spengler, G., Fette und Seifen, *56*, 775 (1954).
26. *Ibid.*, *57*, 474 (1955).
27. *Ibid.*, *57*, 5 (1955).
28. *Ibid.*, *59*, 509 (1957).
29. Trappe, W., Biochem. Z., *306*, 316 (1940).
30. *Ibid.*, *307*, 97 (1941).
31. In press (Olii Min. Grassi Sap. Colori Vern.).

[Received February 15, 1960]