Estimation of Free and Conjugated Glucuronic Acid—Fishman, *et al.*[7] demonstrated the determination of glucuronide in presence of glucuronic acid which is based on the prior oxidation of the acid and the other interfering substances with alkaline hypoiodide solution, the remaining glucuronides being determined by the NR reaction. This procedure provided a valuable method for the estimation of alkali-stable glucuronides in physiological fluids.

The new reagent (NRP), however, was found to be not applicable to the above procedure. Iodide produced by the prior oxidation was oxidized to I_2 during the reaction with the new reagent and interfered in the following color estimation. This difficulty was tolerably well overcome by employing an alkaline Br_2 solution instead of I_2 solution. An example of simultaneous estimation of free and conjugated glucuronic acids (borneol glucuronide) is presented in Table III. Further investigation is being continued.

TABLE III. Estimation of Free and Conjugated Glucuronic Acid (Borneol Glucuronide)

Total acid (T)		Acid (C) in borneol glucuronide		Free Acid (T−C)	
Calcd. (γ/5 cc.)	Found (γ/5 cc.) (%)	Calcd. (γ/5 cc.)	Found (γ/5 cc.) (%)	Calcd. (γ/5 cc.)	Found (γ/5 cc.) (%)
120	118.2 (98.5)	80	78.1 (97.6)	40	40.1 (100.3)
120	119.9 (99.9)	40	39.4 (98.5)	80	80.5 (100.0)
100	101.7 (101.7)	60	61.7 (102.8)	40	40.0 (100.0)
140	143.8 (102.7)	60	60.3 (100.5)	80	83.5 (104.4)

Summary

Naphthoresorcinol picrate, 1:1 molecular compound, is recommended for the microdetermination of glucuronic acid. The new reagent is advantageous over the usual naphthoresorcinol in the stability on storage, both in crystalline and solution state, and it exhibits higher absorbancy and better reproducibility. The standard procedure was established. It has been found that the measurement of glucuronide with this reagent in the presence of glucuronic acid is possible by employing an alkaline bromine method instead of hypoiodide procedure described by Fishman, *et al.*

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88. **Keiichiro Hayashi** : Studies on the Microanalysis of Essential Oil Components. V.[1] Detection of Essential Oil Components by Paper Electrophoresis.

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In the present work, a new method was tried for the electrophoresis of some essential oil components which were regarded hitherto to be difficult to treat by paper chromatography or electrophoresis, since most of them are usually insoluble in water and therefore, it could not be developed on paper by aqueous electrophoresis. A reversed-phase method by silicone-treated or method of chromatography with a paper impregnated with sodium hydrogen sulfite had already been made by the author with special reference to aldehyde compounds.[2] In this respect somewhat similar procedures were applied also in the paper electrophoresis of essential oil components, by

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[1] Part IV. K. Hayashi, Y. Hashimoto : This Bulletin, 5, 618(1957).

[2] K. Hayashi, Y. Hashimoto : *Ibid.*, 5, 74(1957).

which natural liquid alcohols and carbonyl compounds were successfully detected on paper by high-voltage current.

The current of 500~8,000 volts was applied in the experiment for a filter paper strip of 40 cm. in length and it was observed that the migration was often accomplished during around 10 minutes when a current of 3,000~8,000 V was charged, while a few hours was necessary in the experiment by the current of 400~600 V. These methods would be of great aid in the assay and identification of both naturally occuring and synthetically prepared perfumes because of the simplicity of technique.

I) Detection of Terpene Alcohols

1) Anthranilate Method : Chromatographic separation of terpene alcohols converting them into anthranilates has already been described in this series.[3] The same alcohol derivative was used in paper electrophoresis, successfully accomplishing an experiment more rapidly with well separated spots. Alcoholic components in essential oil such as neroli, and jasmin oil were readily identified after converting them into anthraniliate by the procedure already reported.

TABLE I. Migration Distance of Anthranilate

	(1)	(2)
Borneol	−34	−42
Benzyl alcohol	−24	−22
Citronellol	−27	−32
Decyl alcohol	−43	−35
Geraniol	−19	−25
Linalool	−72	−62
Methanol	− 8	−17
Menthol	−53	−55
Nerol	−12	−23

(1) 6*N* AcOH, 1,000 V, 0.35 mA/cm., 2 hrs.
(2) 5*N* AcOH, 1,000 V, 0.45 mA/cm., 2 hrs.

2) Hydrogen Phthalate Method : In the purification of terpene alcohols, the method is available to form hydrogen phthalate of alcohols. These esters were applied to the detection of terpene alcohols, because these could be formed fairly rapidly in a small test tube. The sample of hydrogen phthalate derivatives was dissolved in hexane or ethanol, and spotted on the start line which was drawn in the middle of a filter paper.

TABLE II. Migration Distance of Hydrogen Phthalate

Cinnamic alcohol	+38	Menthol	+33	Geraniol	+23
Citronellol	+18	Nerol	+32		

0.5% $NaHCO_3$, 600 V, 1~1.6 mA/cm., 1 hr.

3) Borate Buffer Method : Some volatile alcohols or phenols were separated by Schöne, *et al.*,[4] in the state of non-volatile boric acid esters, by mixing an equivalent amount of alcohol and boric acid (or its anhydride) to effect ester formation. By the

TABLE III. Migration Distance of Borate

	(1)	(2)	(3)
Citronellol	−13	−41	−16
Geraniol	−10	−28	−39
Linalool	+37	+46	−28
Nerol	−8	−17	−29
Terpineol	+16	+23	−21

(1) 1.2% H_3BO_3 : 2% borax (1 : 1), 600 V, 1~1.7 mA/cm., 1 hrs.
(2) 1.2% H_3BO_3 : 2% borax (1 : 1), 1,000 V, 1~2.2 mA/cm., 30 mins.
(3) 5% H_3BO_3, 800 V, 0.13~0.3 mA/cm., 2 hrs.

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3) T. Kariyone, K. Hayashi : This Bulletin, **4**, 494(1956).
4) A. D. Schöne, O. Zeitchel : D. R. P. 444,640, 448,419(1924).

same procedure, terpene alcohols such as geraniol, citronellol, and linalool were esterified in order to treat them by electrophoresis. The reaction was available generally to the identification of primary, secondary, and tertiary alcohols.

II) Detection of Terpene Aldehydes and Ketones

Sodium hydrogen sulfite electrolyte was recommendable in the electrophoresis of this series of compounds, because a compound with carbonyl groups should conjugate with hydrogen sulfite instantaneously to form its adduct. Sodium hydrogen sulfite was dissolved in 30% propanol solution adjusting the concentration from 0.02% to 0.5%. Instead of the usual filter paper, silicone-treated filter paper(by Dow Corning Silicone 1107)[5] was also used in this experiment and it has shown more or less the same migration distance in every kind of sample. A minute amount of the adduct was enough to spot it on the starting line of the filter paper by dissolving it in ethanol.

TABLE IV. Migration Distance of Terpene Aldehydes and Ketones

	(1)	(2)		(3)	
Cinnamic aldehyde	−27	+24	Hydroxy-citronellal	−4	(No. 52)
p-Tolualdehyde	−26	+37	Hydroxy-citronellal	+9	(Silicone-treated paper)
Citral	−31	+41	Carvone	+12	(Silicone-treated paper)
α-Ionone	−32	+31	Citral	+31	(No. 52)
Carvone	−38	+46	Citral	+21	(Silicone-treated paper)

	(4)		(4)
Octyl aldehyde	+30	Undecyl aldehyde	+16
Nonyl aldehyde	+27	Dodecyl aldehyde	+12
Decyl aldehyde	+25		

(1) 0.02% $NaHSO_3$, 1,000 V, 0.15∼0.2 mA/cm., 1 hr.
(2) 0.5% $NaHSO_3$, 500 V, 1∼1.8 mA/cm., 1 hr.
(3) 0.2% $NaHSO_3$ (30% PrOH), 1,000 V, 0.4∼0.6 mA/cm., 1 hr.
(4) 0.5% $NaHSO_3$ (30% PrOH), 500 V, 0.8∼1.6 mA/cm., 2 hrs.

III) Detection of Aromatic Aldehydes

Aromatic aldehydes such as vanillin, heliotropin, etc. could be separated on paper by electrophoresis as indicated in Table IV, and it was observed that the higher the voltage, the more rapid the migration velocity.

The separation and identification by paper electrophoresis in several to 10 minutes were first realized when a current as high as 8,000 V was charged. In a previous experiment,[6] papergrams were examined by a double-beam, automatic recording densitometer and a document of quantitative curve was obtained to show a linear relationship between the amount of a sample and area corresponding to the spot. Thus, the estimation of aldehyde components in vanilla flavor was readily performed by merely scanning the papergram.

In the present experiment, the electrolyte of sodium hydrogen sulfite solution of concentrations ranging from 0.02% to 0.2% was used, resulting in an interesting migration direction, the phenolic aldehydes migrating to the anode in 0.02% concentration, while these were directed to the cathode when the electrolyte concentration was 0.2%. Consequently, some relations between migration distance and chemical structure were found with special reference to the concentration of sodium hydrogen sulfite electrolyte.

The compound with methoxyl or methylenedioxy group, such as methylvanillin, heliotropin, anisaldehyde, etc., have shown a migration directing to the cathode, while aldehydes with phenolic properties, such as vanillin and ethylvanillin, migrated to the anode in 0.04% concentration.

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[5] K. Hayashi, Y. Hashimoto: This Bulletin, 4, 496(1956).
[6] *Idem.*: *Ibid.*, 5, 74(1957).

Table V. Migration Distance of Aromatic Aldehydes

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Vanillin	+18	−26	−37	−14	−33	−53	−32
Ethylvanillin	+21	−23	−35	−12	−13	—	—
Methylvanillin	+26	−30	−50	+24	+39	+103	+36
Heliotropin	+28	−34	−64	+33	+52	—	+73
Anisaldehyde	+39	−33	−46	+47	+64	—	+131

(1) 0.2% $NaHSO_3$, 1,000 V, 1.4∼2 mA/cm., 1 hr.
(2) 0.02% $NaHSO_3$, 1,000 V, 0.18∼0.23 mA/cm., 1 hr.
(3) 0.02% $NaHSO_3$, 1,500 V, 0.65∼0.8 mA/cm., 2 hrs.
(4) 0.04% $NaHSO_3$, 1,000 V, 0.3∼0.45 mA/cm., 2 hrs.
(5) 0.04% $NaHSO_3$, 1,300 V, 0.8∼1 mA/cm., 2 hrs.
(6) 0.04% $NaHSO_3$, 8,000 V, 3.5∼4 mA/cm., 10 mins.
(7) 0.04% $NaHSO_3$, 3,000 V, 1.2∼1.3 mA/cm., 20 mins.

IV) Eletrophoresis of Coumarins by using Sodium Hydrogen Sulfite

In this respect, Hashimoto, *et al.*[7] has applied a borate buffer separating several hydroxycoumrins. Hydrogen sulfite electrolyte has shown a separation in 0.1% concentration, as listed in Table VI.

Table VI. Migration Distance of Coumarin

Umbelliferone	−15	Coumarin	−9
4-Methylumbelliferone	−16	4,7-Dihydroxycoumarin	−4
Umbelliferone methyl ether	−13		

0.1% $NaHSO_3$, 1,000 V, 0.8∼1.5 mA/cm., 1 hr.

V) Separation of Essential Oils on Paper by Electrophoresis

Aldehyde components of essential oils and volatile natural aldehydes were treated by paper electrophoresis with hydrogen sulfite in hydrous propanol solution. This procedure should be available in the rapid estimation of commercial perfumery products, because the method has made it possible to treat essential oil itself without any preliminary separation or concentration of carbonyl compounds. More or less the same result was obtained when filter paper (Toyo Roshi No. 52) was previously impregnated with hydrogen sulfite followed by drying, although the migration direction of citral was the reverse on water-repellent filter paper treated with a silicone resin.

Table VII. Test of Essential Oils by Electrophoresis

Oil	(1)	(2)
Citronella	+11	+21
Mandariin	+14	+22
Lemonglass	+13	+17
Bergamot	+13	+22
Lime	+14	+17
Lemon (Italian)	+11	+16

(1) 0.5% $NaHSO_3$ (30% PrOH), 600 V, 0.8∼1 mA/cm., 1 hr.
(2) 0.5% $NaHSO_3$ (20% PrOH), 600 V, 1∼1.4 mA/cm., 1 hr.

Table VIII. Electrophoresis of Citral by Specially Treated Paper (Toyo Roshi No. 52)

Without treatment	−17
2% $NaHSO_3$-treated paper	−17
Silicone-treated paper	+16

0.05% $NaHSO_3$ (30% PrOH), 800 V, 0.6∼0.7 mA/cm., 1 hr.

VI) Paper Electrophoresis of Aldehydes and Lactones using Cadmium or Mercuric Chloride

Recently, Marini Bettolo, *et al.*[8] introduced a new reaction to the detection of aldehydes, ketones, and lactones in which adducts of these carbonyl compounds with

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7) Y. Hashimoto, I. Mori, M. Kimura : "Device for Paper Electrophoresis," 41.
8) G. B. Marini Bettolo, Leonello Paolini : Gazz. chim. ital., **75**, 78(1945).

mercuric or cadmium chloride were formed. The adduct formation was applied to the electrophoresis on paper using mercuric chloride solution as the electrolyte. The separation of these groups of compounds on paper was effected as indicated in Table IX.

TABLE IX. Migration Distance by Mercuric Chloride Method

	(1)		(2)		(3)
Vanillin	−20	Umbelliferone	−13	Ethylvanillin	−15
Umbelliferone	−15	Umbelliferone methyl ether	−15	Methylvanillin	−17
Coumarin	−29	4-Methylumbelliferone	−15	Vanillin	−25
Citral	−26	Coumarin	−22	Heliotropin	−20
				Anisaldehyde	−26

(1) 0.1% $HgCl_2$, 1,000 V, 0.15∼0.35 mA/cm., 1 hr.
(2) 0.4% $HgCl_2$, 1,000 V, 0.22∼0.3 mA/cm., 1 hr.
(3) 0.04% $HgCl_2$, 1,000 V, 0.3∼0.7 mA/cm., 1 hr.

VII) Detection of Spots of Terpene Alcohols, Aldehydes, Ketones, and Lactones on Filter Paper

Microidentification of essential oil components by detection of spots on paper with miscellaneous coloring reagents or by using ultraviolet light has already been described. In the light of diversities of color tests, these are classified below to facilitate finding which test will be the most satisfactory in detecting spots on paper.

1) Alcohol anthranilates[9]:
   a) Ehrlich reagent
   b) $SbCl_5$, 10% $CHCl_3$ solution
   c) Ultraviolet light
2) Alcohol hydrogen phthalates:
   a) Fluorescein-bromine reagent[10]
   b) Eosin, 0.001% solution
   c) $SbCl_5$, 10% $CHCl_3$ solution
3) Alcohol and phenol borates:
   a) Fluorescein-bromine reagent
   b) Eosin, 0.001% solution
   c) $SbCl_5$, 10% $CHCl_3$ solution
4) Aldehydes and Ketones:
   a) 2,4-Dinitrophenylhydrazine, 0.4% solution in $2N$ HCl
   b) Ehrlich-Müller reagent[11]
   c) 5% Sodium nitroprusside solution, 2% NaOH solution[12]
   d) *m*-Dinitrobenzene, 2% solution, 1% NaOH solution[13]
   e) *m*-Phenylenediamine, 1% solution[14]
   f) *p*-Phenylenediamine, 2% solution, 3% H_2O_2 and $2N$ AcOH solution
5) Lactones:
   a) Fluorescence under ultraviolet rays

The author is grateful to Dr. Tatsuo Kariyone, Director of National Institute of Hygiene, for encouragement in carrying out this study, and also to Prof. Dr. Yohei Hashimoto, Kobe College of Pharmacy, for kind advices.

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[9] Part I. T. Kariyone, K. Hayashi: This Bulletin, **4**, 494(1956).
[10] J. Kirchner, *et al.*: Anal. Chem., **23**, 420(1951).
[11] H. Müller: Chem. Ztg., 673(1951).
[12] v. Bitto: Ann., **267**, 372(1892).
[13] *Ibid.*, **269**, 377(1892).
[14] v. Bitto: Bull. **36**, 371(1897).

Experimental

Paper Electrophoresis—A constant-voltage paper electrophoresis apparatus Model III supplied by Toyo Roshi Co. Ltd. was used throughout the experiments. 1% EtOH solution of the sample was spotted on the starting line in the center of a filter paper with a micropipette to deposit 0.01~0.03 cc. of solution.

Terpene Alcohol Hydrogen Phthalate—A sample of essential oil composed of terpene alcohol or aromatic or aliphatic alcohol, together with the same volume of benzene and equivalent amount of phthalic anhydride was boiled gently on a water bath for 2~3 hrs. The concentrated sample in benzene was diluted so as to make 1% hydrogen phthalate solution and spotted on the starting line of the filter paper.

Preparation of Silicone-treated Paper and Paper impregnated with Sodium Hydrogen Sulfite—A filter paper strip (Toyo Roshi No. 52, 2×40 cm.) was immersed in 5% benzene solution of Dow Corning Silicone No. 1107 and excess solution was removed by pressing it with a rubber roller between two sheets of dry filter paper. After evaporation of the solvent, the paper was heated at 120° for 1 hr. to complete the polymerisation of resin on the cellulose fibre.

To prepare the bisulfite-treated paper, a paper strip was immersed in 2% $NaHSO_3$ solution and excess solution was removed in the same way and dried.

Preparation of Color Reagents—(1)For the detection of alcohols : The following spray reagents are recommendable.

Ehrlich-Müllers reagent : By alternative spraying of 5% AcOH solution of *p*-dimethylaminobenzaldehyde and conc. H_3PO_4. Anthranilates show orange red coloration.

$SbCl_5$, 10% $CHCl_3$ solution : The anthranilate colors orange red.

Ultraviolet ray : The location of spot on paper could be distinguished by irradiation of ultraviolet ray of 3650 Å.

Fluorescein-bromine reagent : The reagent is also available for compounds with ethylenic double bond, revealing yellow spots.

Spraying of 0.001% EtOH solution of eosin or umbelliferone : By applying a fine mist of eosin and umbelliferone solution, dark spots of hydrogen phthalate is distinguished on a fluorescent background by the irradiation of ultraviolet ray of 2537 Å. In the case of umbelliferone reagent,

TABLE X. Color of Spot

	KOH, U.V.	*m*-Phenylenediamine	*m*-Phenylenediamine +U.V.	*p*-Phenylenediamine with H_2O_2	Na nitroprusside	*m*-Dinitrobenzene
Vanillin	Dark violet	Yellow	Red brown	Yellow brown	Violet brown	Yellow
Methylvanillin	Dark violet	Brown	Violet	Yellow	—	Brown
Ethylvanillin	Dark violet	Yellow	Red brown	Yellow brown	Violet brown	Yellow
Coumarin	Yellow green	—	—	—	—	—
Umbelliferone	Blue	—	—	—	—	—
Heliotropin	—	Brown	Dark violet	Yellow	Red purple	—
Octyl aldehyde	—	Pale yellow	Pale yellow	Brown→violet	Brown	Purple
Nonyl aldehyde	—	Yellow brown	Pale yellow	Brown→violet	Red purple	Red purple
Decyl aldehyde	—	Yellow brown	Yellow green	Blue green →violet	Red purple	Purple
Undecyl aldehyde	—	Yellow	Yellow	Green→violet	Violet	Red purple
Dodecyl aldehyde	—	Yellow brown	Yellow green	Green→violet	Red purple	Red purple
Citral	Dark violet	Yellow brown	Dark violet	Yellow	Red purple	Violet
α-Ionone	Dark violet	Brown	Red violet	Pale yellow	Violet	Orange red
p-Tolualdehyde	Dark violet	Yellow brown	Dark violet	Pale yellow	Red purple	—
Anisaldehyde	Dark violet	Yellow brown	Violet	Yellow	Violet brown	—
Carvone	Dark violet	Brown	Dark violet	Brown	Violet	Yellow brown
Hydroxycitronellal	Dark violet	Orange red	Red violet	Red purple	Yellow	Brown

alternative spraying of 0.2% caustic alkali solution and umbelliferone solution was desirable. It was readily obtained by the ultraviolet ray of 2537 Å, by attaching Kaken 2537 Å filter on the disinfection light tube (Mazda GL-15), by which detection of spots on paper was more sensitive than that by the commercial black light tube of 3650 Å.

10% $CHCl_3$ solution of $SbCl_5$: All phthalates of terpene alcohols turned yellowish brown to orange yellow.

Color reagents for borate buffer electrolyte for terpene alcohol: The same reagents are available as for alcohol hydrogen phthalates mentioned above.

(2) **Aldehydes, Ketones, and Lactones**—Miscellaneous color reagents were used for revealing spots of aldehydes, ketones, and lactones on filter paper, including ultraviolet ray (Table X), which is one of the most sensitive detection as was pointed out by Feigl with coumarin and its derivatives. In general, 2,4-dinitrophenylhydrazine, *m*- and *p*-phenylenediamine, *m*-dinitrobenzene, and sodium nitroprusside showed some coloration with aldehydes and ketones, but none was observed for coumarin, umbelliferone, and related compounds.

Summary

Extensive studies were made for the detection of terpene alcohols, aldehydes, ketones, and lactones by paper electrophoresis. The method is generally available for a rapid test of perfume products and it could be accomplished more rapidly than chromatography on paper by using a current of 500~8,000 V, reported previously. The higher the voltage, the sooner the results are obtained in electrophoresis, while a constant time was required in chromatography owing to the constant capillarity of the filter paper itself. One of many electrolytes applied, mercuric chloride, has shown the most satisfactory result and a formation of conjugated carbonyl groups with mercuric ion was presumed.

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89. **Kiichi Arakawa and Masachika Irie**: Bromination of Cyclic 1,3-Diketone Monoenolates with N-Bromosuccinimide. I.

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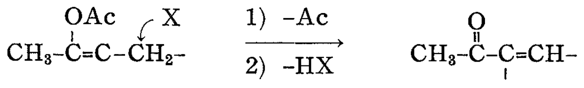
Several results have been reported on the bromination of an alkyl or acyl enolate and these could be divided into two groups from the type of their reaction. Thus, 3α,12α,20-triacetoxypregn-17-ene and 3β,20-diacetoxyallopregn-17-ene were reacted with N-bromosuccinimide (NBS) in the presence of peroxide and respectively gave 3α,12α-diacetoxypregn-16-en-20-one and 3β-acetoxyallopregn-16-en-20-one.[1]

In these reactions allyl bromination with NBS at C-16 was followed by deacetylation and dehydrobromination with simultaneous rearrangement of the double bond to give the final product. The general type of these reactions could be represented by (A).

(A)



$$CH_3-\overset{\overset{OAc}{|}}{C}=C-\overset{X}{CH_2}- \xrightarrow[2)\ -HX]{1)\ -Ac} CH_3-\overset{\overset{O}{\|}}{C}-\underset{|}{C}=CH-$$

Another type of the reaction is summarized as (B) in which 2-hepten-2-ol acetate, pregna-5,20-diene-3β,20-diol diacetate, and pregna-5,16,20-triene-3β,20-diol diacetate were reacted with NBS or N-iodosuccinimide (NIS) to furnish 3-bromoheptan-2-one, 3β-acetoxy-21-iodopregn-5-en-20-one, and 3β-acetoxy-21-iodopregena-5,16-dien-20-one

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[1] C. Djerassi, C. R. Scholz: J. Org. Chem., **14**, 660(1949).