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Color development of proanthocyanidins in vanillin-hydrochloric acid reaction*

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Abstract The influence of proanthocyanidin (PA) structures contained in bark on color development in the vanillin-hydrochloric acid (V-HCl) method used widely as a quantitative method for measuring PA were examined. The maximal absorption wavelength was different in terms of the bark from which the PA was obtained. Phenyl nucleus (resorcinol, phloroglucinol) constituting the A-ring of PA reacts with vanillin to produce the color. The maximal absorption wavelengths of the solutions from synthesized procyanidin and profisetinidin were 500 and 540 nm, respectively, indicating that the color tone differs in the V-HCl method based on the hydroxylation patterns of the A-ring. The colored solution of (+)-catechin with vanillin was dialyzed, and the resulting product (C-VC) was analyzed by gel permeation chromatography and ^{1}H nuclear magnetic resonance. It was found that C-VC was a polymer complex consisting of 9 mol (+)-catechin moieties and 10 mol vanillin moieties. It was presumed that the cationized vanillin molecules that do not combine with (+)-catechin play an important role on color development in the presence of C-VC.

Key words Proanthocyanidin · Vanillin-HCl method · (+)-Catechin–vanillin complex · Co-pigmentation

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Introduction

When a monomeric or polymeric flavan-3-ol reacts with vanillin under acidic conditions, a red color develops with an absorption maximum at 500 nm. This vanillin-hydrochloric acid (V-HCl) method is widely used for rapid, simple quantitative measurement of proanthocyanidins (PAs) in plant materials. This method has been especially developed by determining condensed tannins contained in sorghum grains,[1–3] and some modifications have been devised because of its stability and the reproducibility of its coloration.[4,5] The method is also applied to determine the PA content in some coniferous barks[6] and is widely used in the analytical method for polyphenols as well as the Folin Denis[7] and Lowenthal methods.[6] The question has been raised whether this method can be applied to PAs except for the procyanidin (PC) type (e.g., the profisetinidin and prorobinetinidin types found in quebracho and wattle extracts) because it is a colorimetric determination based on the color development of (+)-catechin, monomeric PC.

In this study we examined the influence of the structures of PAs on color development. The structure of the (+)-catechin–vanillin complex relating to color development in the V-HCl method is also discussed.

Materials and methods

Proton nuclear magnetic resonance ^{1}H-NMR spectra were measured on a JMN-A500 spectrometer. The infrared (IR) absorption spectrum was measured by a Shimazu IR-470 spectrophotometer.

Materials

Commercially available resorcinol, phloroglucinol, catechol, and pyrogallol were used as model compounds of the phenyl nucleus constituting PA. PAs from karamatsu (*Larix leptolepis*) bark, acacia (*Acacia mearnsii*) bark, and

commercially available wattle (*Acacia* sp.) and quebracho (*Schinopsis lorentzii*) extracts were fractionated by Sephadex LH-20 column chromatography according to our previous paper.[8] PAs of the PC and PF type oligomers were synthesized by condensation of the corresponding flavan-3,4-diols with (+)-catechin under acidic conditions. (+)-Taxifolin and (+)-fustin were obtained from the heartwood of karamatsu (*Larix leptolepis*) and hazenoki (*Rhus succedanea* L), respectively.

Synthesis of oligomeric PAs

Isolation of flavanonol

(+)-Taxifolin. The tree sampled for (+)-taxifolin was a 30-year-old karamatsu growing in the Mie University Forest (Misugi, Mie) in August 1990. The heartwood sample was ground in a Wiley mill. The air-dried heartwood meal (1000 g) was extracted with methanol (10 l) for 24 h at ambient temperature. After evaporating the methanol, the residue was extracted with ether (500 ml). The ether solution was evaporated to about 100 ml under reduced pressure, and the concentrated solution was stored in a refrigerator. Afterward, a 5 g of the ether-soluble part was separated into four fractions by column chromatography on a Sephadex LH-20 gel (2.5 × 80.0 cm) using ethanol as an eluent. Crude crystal (1.0 g) was isolated after several recrystallizations of the second fraction from hot water. It was identified by co-thin layer chromatography (TLC) and co-high-performance liquid chromatography (HPLC) with an authentic sample.

(+)-Fustin. The tree sampled for (+)-fustin was an 80-year-old hazenoki growing in Wakayama Prefecture in spring 1992. The heartwood sample was ground in a Wiley mill. The air-dried heartwood meal (500 g) was extracted with methanol (7 l) for 24 h at ambient temperature. After evaporating the methanol, the residue was extracted with ether (500 ml). The ether solution was evaporated to about 100 ml under reduced pressure, and the concentrated solution was stored in a refrigerator. Afterward, a portion (5 g) of the soluble ether was separated into three fractions by column chromatography on a Sephadex LH-20 gel (2.5 × 80.0 cm) using ethanol as an eluent. Crude fustin crystal (1.3 g) containing a small amount of fisetin was isolated. Fustin was identified by co-TLC and co-HPLC with an authentic sample.

Preparations of synthesized PAs. Flavanonols (500 mg) in ethanol (100 ml) solution was stirred with $NaBH_4$ (500 mg) for 4 h. The yellowish reaction mixture was poured into an excess of water, and the pH was adjusted to 3–4 with phosphoric acid. The solutions was extracted with ethyl acetate (100 ml × 3), and the combined extract was dried over Na_2SO_4. Because flavan-3,4-diols (leucocyanidin and leucofisetinidin) are known to be highly labile compounds under acidic conditions, the extract was deacidified and chromatographed on a LH-20 gel column using water and ethanol as eluents, respectively. These flavan-3,4-diols were obtained in yields of 390 and 425 mg, respectively. Flavan-3,4-diols (160 mg) and (+)-catechin (160 mg) were dissolved in 0.1N HCl (20 ml), and the mixture was stirred at 25°C for 24 h. The reaction solution was then deacidified on a Sephadex LH-20 column (2 × 45 cm) eluted with water and subsequently chromatographed on the same column eluted with ethanol, methanol, and 70% aqueous acetone solution, respectively. The methanol eluate fraction was collected and freeze-dried, and amorphous brownish yellow compounds were obtained.

V-HCl method

Methanol solution (1 ml) containing 0.8 mg of sample was placed in a brown tube to which 6 ml of methanol solution containing 4% vanillin was added. After stirring vigorously, 3 ml of concentrated hydrochloric acid was added to the solution. After stirring with a tube mixer every 5 min for 15 min, the absorbance at 400–600 nm of the red solution was measured by a spectrophotometer (Jasco V-520).

Isolation of (+)-catechin–vanillin complex

(+)-Catechin (100 mg) was dissolved with 2.8 ml of 4% vanillin methanolic solution in a brown tube to which 1.2 ml of concentrated HCl was added. The tube was stirred vigorously for 15 min and the resulting colored solution was poured into a cellophane tube (MW 1000 daltons) and dialyzed overnight against 1000 ml of deionized water. The sample solution dialyzed was freeze-dried, and the resulting powder was dissolved in a small volume of methanol. The solution was poured into excess ether to remove the vanillin entirely. The resulting precipitates were collected by centrifugation and dried over P_2O_5 to obtain an amorphous brownish (+)-catechin–vanillin (C-VC) powder (110 mg). The V-HCl method for determining C-VC was conducted according to the procedure described above.

Gel permeation chromatography

Gel permeation chromatography (GPC) was recorded by a Jasco Trirotar system with Shodex GPC columns KF-802 and KF-804 (4.6 mmϕ × 250 mm) using tetrahydrofuran as an eluent. The chromatogram was calibrated against standard polystyrenes (molecular weights 2000 and 9000 daltons), (+)-catechin, and synthesized profisetinidin (PF) dimer. Average molecular weights ($\bar{M}n$, $\bar{M}w$) were calculated by an integrator, Jasco 807-IT, from the molecular weight distributions obtained by the GPC measurement.

Phenyl nucleus exchange reaction

The phenyl nucleus exchange reaction was carried out in a 2-ml glass ampule with 10 mg polyphenol sample and 500 µl degradation reagent at a temperature of 80° or 150°C. The constitution of the degradation reagent was benzene:phenol:BF_3–phenol complex (10:19:3, v/v). After

the reaction the reaction vessel was cooled to room temperature and the reaction mixture transferred quantitatively to a beaker with ethyl ether. A known amount of internal standard (dibenzyl in benzene) was added. Ether-insoluble materials were filtered and washed with ethyl ether. The filtrate and washings were combined in a separatory funnel to which an excess of saturated brine was added. After the extraction (30 ml × 3), the ether layer was dried over sodium sulfate and evaporated to a small volume (4–5 ml). A 100-μl portion of the ether solution was transferred to a small vial to which a few drops of pyridine and 100 μl *N,O*-bis(trimethylsilyl) acetamide were added. After a 1-h derivatization reaction at room temperature, the TMS derivatives were analyzed quantitatively using gas-liquid chromatography (GLC). GLC was performed by a Yanagimoto G-180 using a methyl silicone capillary column (Guadrex S2006, 0.25 mm i.d., 25 m length × 0.25 mm film thickness). The column temperature of the GLC was maintained at 80°C for 2.5 min and then increased to the final temperature of 250°C at a rate of 2°C/min.

Results and discussion

V-HCl reaction of bark extracts

Figure 1 shows the absorption spectra of the reaction products of the bark extracts by the V-HCl reaction at 400–600 nm. The maximal absorption wavelength (λmax) of the product from karamatsu is about 500 nm, whereas those of other species are 530–540 nm. In general, calculation of a flavanol value is based on the connection between the concentrations of (+)-catechin and its absorbance at 500 nm. As described above, however, a bathochromic shift against 500 nm of the colored solution of V-HCl reaction in some species is observed, so particular attention should be paid to quantification of PA by the V-HCl method. The analysis of constituent nucleus of the PA contained in the each extracts by the nucleus exchange reaction is shown in Table 1.

Karamatsu extracts liberate phloroglucinol and catechol, and quebracho extracts liberate resorcinol and catechol, respectively, in the reaction. These results indicate that the PAs of karamatsu extracts consist of the PC type and those of quebracho extracts the PF type. Acacia extracts, on the other hand, consist of prorobinetinidin (PR) and PF types mainly because resorcinol is liberated primarily from the A-ring and catechol and pyrogallol from the B-ring. Therefore the intimate relations between color development with the V-HCl method and hydroxylation patterns on the A-ring of PA are presumed.

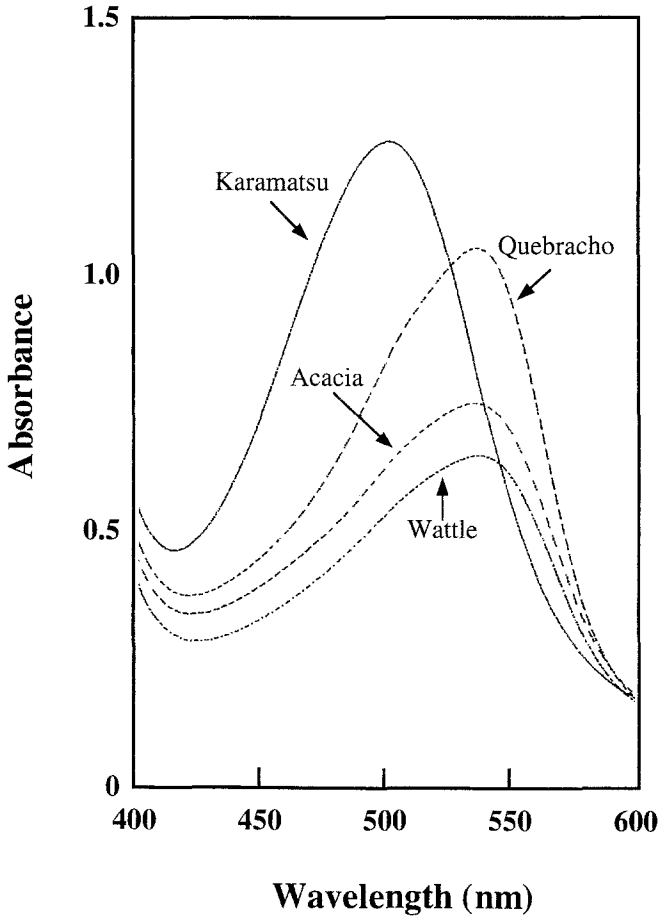

Fig. 1. Absorption spectra of the colored solution from 70% aqueous acetone extracts of bark by the vanillin-HCl method. The reaction was carried out with a concentration of 0.8 mg per ml of methanolic solution of each extract

Table 1. Nuclei liberated from bark extracts by the nucleus exchange reaction

Sample	A-ring (mol%)[a]		B-ring (mol%)[a]	
	Phl.	Res.	Cat.	Pyr.
A-ME	15.1	49.5	22.1	38.1
A-AE	11.3	33.1	17.3	35.6
Q-ME	5.3	40.6	64.4	2.0
Q-AE	3.3	43.3	63.6	2.3
K-ME	20.5	0.5	65.0	2.8
K-AE	23.4	0.6	70.3	3.1

A, acacia; Q, quebracho; K, karamatu; ME, methanol eluate fraction; AE, 70% aqueous acetone eluate fraction; phl., phlorogrucinol; res., resorcinol; cat., catechol; pyr., pyrogallol.
[a]These values are represented by mol% to a unit of flanan-3-ol.

Color development of model compounds and synthesized PA

The V-HCl method of model compounds of phenyl nuclei constituting PAs was examined to confirm the relations of the color development and the structural factors of the PAs. The gram absorptivity (*a*) at 500 nm and maximal absorption wavelength (λmax) of the color developed in the solutions of these compounds are shown in Table 2. The *a* value of resorcinol or phloroglucinol constituting the A-ring phenyl nucleus was higher than that of pyrogallol or catechol constituting the B-ring. Furthermore, the bathochromic

Table 2. Maximal absorption wavelength and gram absorptivity of model compounds of phenyl nuclei constituting proanthocyanidins

Model compounds	λmax (nm)	a^a
Phenol	–	0
Catechol	–	0
Pyrogallol	530	1.3
Resorcinol	530	6.5
Phloroglucinol	487	14.3

λmax, maximal absorption wavelength; *a*, absorptivity.
[a] Absorptivity was calculated from absorbance at 500 nm.

Table 3. Maximal absorption wavelength and gram absorptivity of synthesized proanthocyanidins

MW[a]	λmax (nm)	a^b
Profisetinide type		
580	544	0.70
1200	547	0.25
1700	548	0.23
3300	543	0.20
Procyanidin type		
290	500	3.42
590	501	2.58
1050	501	2.31
2500	502	2.05
2700	500	1.76

See Table 2 for explanation of abbreviations.
[a] Molecular weight calculated from the elution volume of top peak of gel permeation chromatography.
[b] Absorptivity was calculated from absorbance at 500 nm.

shift of the color developed from resorcinol was observed and compared with that from phloroglucinol. These results are in fair agreement with the data obtained by Goldstein and Swain.[9]

Table 3 shows the λmax and *a* values of the solutions from synthesized PAs with different average molecular weights. The λmax of the PF type was 543–548 nm, and that of the PC type was 500–502 nm. Taking into account that the synthesized PF types have fisetinidol in extender units and a catechinyl structure in the initial unit, this bathochromic shift of the PF type would be due to the resorcinol A-ring in extender units. Additionally, the *a* value of the PC type was about 10 times higher than that of the PF type, indicating that the reactivity of the PC type with vanillin is fairly high compared with that of the PF type under acidic conditions as observed in the reaction of PAs with formaldehyde reported in our previous paper.[8] The *a* value also decreased with the increase in molecular weight for both types. It is therefore apparent that the degree of color development with the V-HCl method changes largely in terms of the hydroxylation patterns on the A-ring and the molecular weight of the PA.

Structure of the (+)-catechin–vanillin complex

The color development of the PA using the V-HCl method, in general, is understood to occur through quinonemethide structures of the PA–vanillin complex, binding a vanillin to the C6 or C8 position on an A-ring of PA.[10] The data relating to the structure of colored products, however, have rarely been found. Thus the structure of C-VC is examined

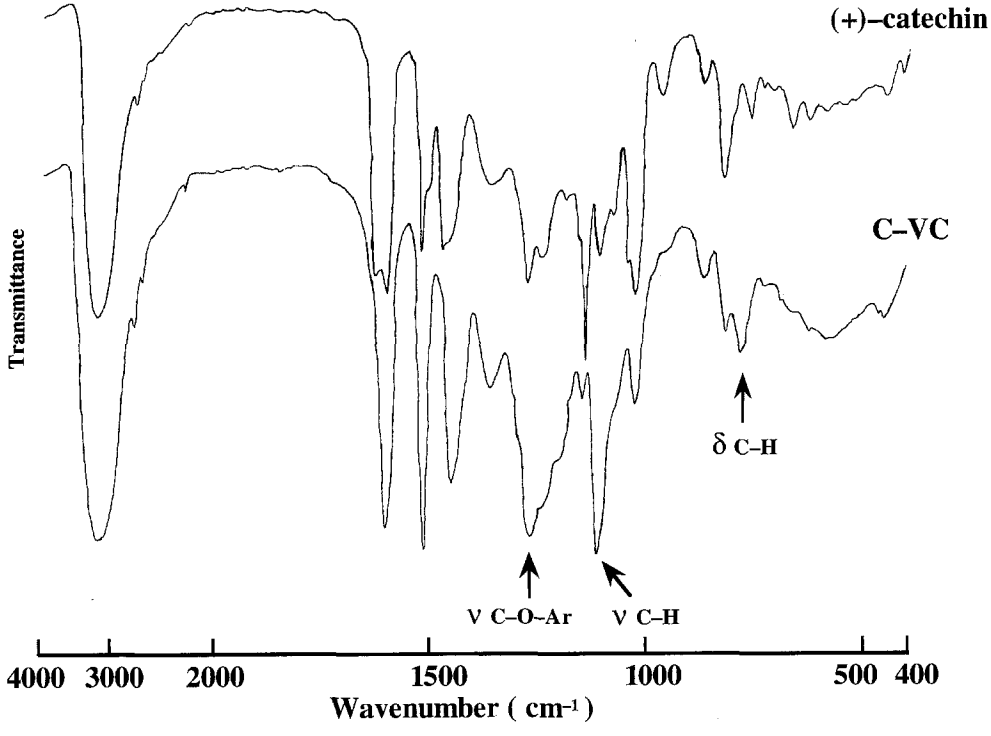

Fig. 2. IR spectra of (+)-catechin and (+)-catechin-vanillin complex (C-VC). δ*C-H*, deformation band; ν*C-H*, ν*C-O-Ar*, stretching bands from guaiacyl ring and arylether, respectively

to obtain information about the color development mechanism of PAs with the V-HCl method.

The IR spectrum of C-VC and the ^{1}H-NMR spectrum of its acetate are shown in Figs. 2 and 3, respectively. The deformation ($\delta_{C\text{-}H}$) band originated from vicinal protons on 1,2,4-substituted benzene and the stretching ($\nu_{C\text{-}H}$) and ($\nu_{Ar\text{-}O\text{-}C}$) bands from guaiacyl ring and arylether appeared at 780, 1120, and 1270 cm^{-1}, respectively; that the sretching ($\nu_{C=O}$) band from the carbonyl group of vanillin did not appear at 1670 cm^{-1}, suggests that the condensation reaction takes place between the carbonyl carbon in vanillin and C6 or C8 of (+)-catechin. Furthermore, the broad signal at δ 3.5–3.9 ppm assigned to the methoxyl group was observed in the ^{1}H-NMR spectrum, supporting the above suggestion. The amounts of combined vanillin in C-VC were calculated using the ^{1}H-NMR spectrum as follows. If x mol of vanillin combines with 1 mol of (+)-catechin, peak areas due to the methoxyl protons and the phenolic acetoxyl protons should be counted ($3x$) and ($3x + 12$), respectively, corresponding to the integration value at δ 3.5–3.9 ppm and that at δ 2.1–2.4 ppm. The equation $(3x):(3x + 12) = 3:13.81$ holds, so x is estimated at 1.11. Furthermore, it has been made clear that the average molecular weight of C-VC is estimated at 4100 daltons by GPC analysis. Therefore it can be assumed that C-VC consists of nine catechinyl units and ten 3-methoxy-4-hydroxyphenyl units on average.

Effect of vanillin on color development

The amorphous C-VC is brownish yellow, which is presumed to be related to the compound during the color development of (+)-catechin using the V-HCl method. Table 4 shows the λmax and a value of C-VC when concentrated HCl or concentrated HCl and vanillin were added to the C-VC methanolic solution. The C-VC solution to which concentrated HCl was added showed 464 nm of λmax and a low a value, whereas a bathochromic shift to 499 nm was observed when vanillin was added to the C-VC solution containing concentrated HCl, which indicates that the vanillin molecules that do not combine with (+)-catechin contribute to the red color development. The relations between the a value and λmax, respectively, and the vanillin concentration in the colored solution are shown in Fig. 4. The a value increases with increasing vanillin concentration, whereas the λmax of the colored solution changes slightly in a vanillin concentration of more than 2% and shifts drastically to a short wavelength in a concentration lower than that. This phenomenon seems to be exhibit behavior similar to that of the co-pigmentation effects,[11] which are closely related to the development of color in flowers, forming complexes between anthocyanins and flavones.

The color development of PAs using the V-HCl method probably takes place as follows. A condensation reaction occurs between the C6 or C8 position of some flavan-3-ol units in PA and the carbonyl carbon of vanillin. As a result, a cross-linked polymer is produced. Vanillin molecules that do not take part in the cross-linking reaction may be cationized in the presence of a strong acid associated with

Table 4. Color development of C-VC by the vanillin-HCl method

Sample	λmax (nm)	a (1/g cm)
(+)-Catechin		
Conc. HCl	–	–
Conc. HCl + vanillin	500	31.1
C-VC		
Conc. HCl	464	0.9
Conc. HCl + vanillin	498	16.3

Reaction was carried out with a 1.0 mg/ml concentration of sample in methanolic solution.
C-VC, (+)-catechin–vanillin complex; see Table for explanation of other abbreviations.

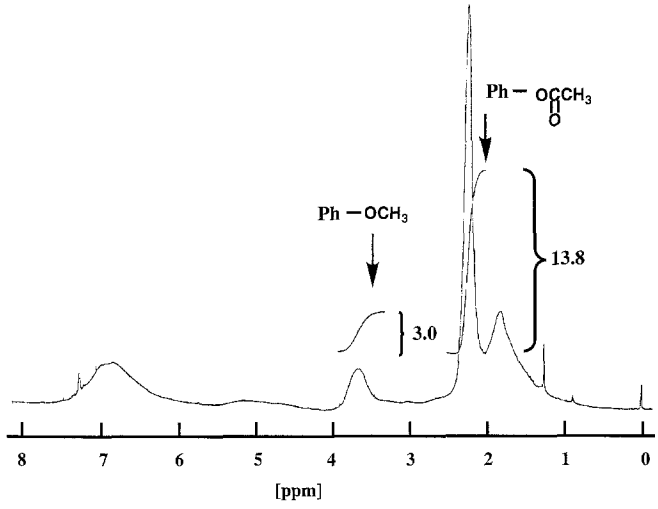

Fig. 3. ^{1}H-NMR spectrum of C-VC acetate

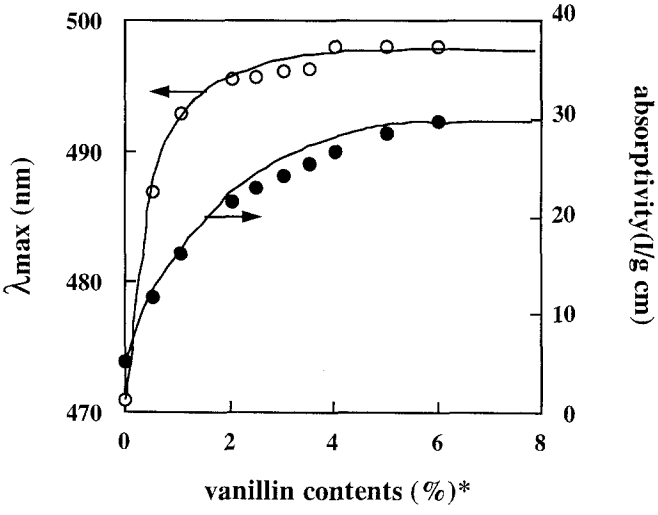

Fig. 4. Effect of vanillin concentration on the color development of C-VC in the vanillin-HCl reaction. The reaction was carried out in a test tube containing 0.9 mg of sample in 3 ml of methanol, the prescribed amount of vanillin, and 1 ml of concentrated HCl. *Vanillin contents (%) represents the weight proportion of 3 ml of methanol solution

the polymer. A charge transfer then occurs between the two molecules to produce the color.

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