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# Determination of hydroxycinnamic acids, hydroxybenzoic acids, hydroxybenzaldehydes, hydroxybenzyl alcohols and their glucosides by high-performance liquid chromatography

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## Abstract

Three 4-hydroxycinnamic acids, three 4-hydroxybenzoic acids, three 4-hydroxybenzaldehydes, three 4-hydroxybenzyl alcohols and their glucosides were separated by HPLC. HPLC analyses were performed with an octadecyl silica gel column and a gradient elution with methanol and water adjusted to pH 4.6 or 3.1. Compounds extracted from *Vanilla planifolia* green beans were analyzed by this method and 4-hydroxybenzaldehyde, vanillin and their respective glucosides were determined.

**Keywords:** Hydroxycinnamic acid; Hydroxybenzoic acid; Hydroxybenzaldehydes; Hydroxybenzyl alcohols

## 1. Introduction

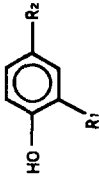
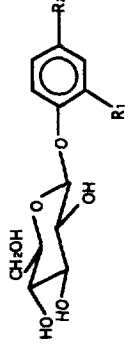
Recently, many studies on glycosides as flavor or fragrance precursors have been done [1–14]. It is known that flavor or fragrance substances are released after hydrolysis of their glycosides by enzymes in plants. As an example of flavor precursors, vanillin is well known to be formed from glucovanillin, a glucoside of vanillin, by  $\beta$ -glucosidase [11–14]. Although glucovanillin seems to be derived from phenylpropanoids via their glucosides [13,15,16], a biosynthetic pathway showing how glucovanillin can be obtained from phenylpropanoids has not yet been clarified. We have started an investigation on precursors of glucovanillin in order to demonstrate a whole pathway of vanillin biosynthesis.

Analytical studies of glycosides, glucose esters or quinic esters of hydroxycinnamic acids by HPLC have been reported [17–22]. Good separations were achieved using ODS columns. However, our study required complete separation of glucosides of 4-hydroxyphenyl compounds ( $C_6-C_1$ ) which are related to vanillin biosynthesis, as well as glucosides of 4-hydroxycinnamic acids ( $C_6-C_3$ ) as shown in Fig. 1. This pathway was proposed on the basis of other reports [12–16]. It is necessary to analyze native glucosides in plants without first hydrolyzing them to aglycones [14] and to demonstrate their conversions in vivo. However, there is no report on the simultaneous separation of the above compounds. Prior to our study on vanillin biosynthesis, we investigated the optimum conditions for separation of the 24 compounds related to vanillin biosynthesis by HPLC. This paper deals with the results of the separation. The analytical method was further ap-

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Table 1  
Authentic compounds

Aglycone		Glucoside*	
			
R <sub>1</sub>	R <sub>2</sub>		
	CH=CHCOOH	COOH	CH <sub>2</sub> OH
H	<i>p</i> -Coumaric acid <sup>b</sup> (4-Hydroxycinnamic acid)	4-Hydroxybenzoic acid	4-Hydroxybenzyl alcohol
OH	Caffeic acid (3,4-Dihydroxycinnamic acid)	Protocatechuic acid (3,4-Dihydroxybenzoic acid)	Protocatechualdehyde (3,4-Dihydroxybenzaldehyde)
OCH <sub>3</sub>	Ferulic acid (4-Hydroxy-3-methoxycinnamic acid)	Vanillic acid (4-Hydroxy-3-methoxybenzoic acid)	Vanillyl alcohol (4-Hydroxy-3-methoxybenzyl alcohol)

\* Names of glucosides are made by addition of “β-D-glucoside” to names of aglycones.

<sup>b</sup> In this table only the names of aglycones were written.

alcohol, vanillyl alcohol, 4-hydroxybenzoic acid- $\beta$ -D-glucoside, 4-hydroxybenzyl alcohol- $\beta$ -D-glucoside, 4-hydroxybenzaldehyde- $\beta$ -D-glucoside, vanillic acid- $\beta$ -D-glucoside, vanillyl alcohol- $\beta$ -D-glucoside and glucovanillin were kindly provided by Takasago International Corporation (Tokyo). *p*-Coumaric acid- $\beta$ -D-glucoside, caffeic acid- $\beta$ -D-glucoside [4-( $\beta$ -D-glucopyranosyloxy)-3-hydroxycinnamic acid], ferulic acid- $\beta$ -D-glucoside, protocatechuic acid- $\beta$ -D-glucoside [4-( $\beta$ -D-glucopyranosyloxy)-3-hydroxybenzoic acid] and protocatechualdehyde- $\beta$ -D-glucoside [4-( $\beta$ -D-glucopyranosyloxy)-3-hydroxybenzaldehyde] were synthesized by Michael's method, which is based on the fact that  $\alpha$ -acetobromoglucose reacts with the respective aglycone in an alkaline  $H_2O/Me_2CO$  solution [23]. Acids were glucosylated after their esterification with MeOH. Deacetylation of the sugar moiety was performed with NaOMe and hydrolysis of the acid methyl ester with  $Na_2CO_3$ . Protocatechualcohol and protocatechualcohol- $\beta$ -D-glucoside [4-( $\beta$ -D-glucopyranosyloxy)-3-hydroxybenzyl alcohol] were obtained by the reduction of protocatechualdehyde and protocatechualdehyde tetraacetyl- $\beta$ -D-glucoside with  $NaBH_4$ , respectively. The structures of the synthetic compounds were confirmed by measuring their NMR spectra with a JNM-A400 spectrometer (JEOL, Japan).

## 2.2. Sample extraction and prepurification

A 5-g amount of *Vanilla planifolia* green beans (6 months after pollination), which were cultivated in Indonesia, was cut into small pieces, suspended in 80 ml of methanol and homogenized. After standing for 1 h at room temperature, the plant pulp was filtered. The residue was re-extracted with 80 ml of 80% MeOH. The combined extracts were concentrated to less than 1 ml in a rotary vacuum evaporator at 40°C. After addition of 20 ml of water, the residual aqueous solution was extracted three times with 10 ml of *n*-pentane. Aqueous solution was then extracted five times with 20 ml of ether. The ether fraction contained aglycones. After the ether was evaporated, the extracts were dissolved and made up to a 5-ml volume with 50% MeOH. The aqueous solution was concentrated to remove ether and applied to Amberlite XAD-2 column ( $\phi$  2.0×17.0

cm). The column was washed with water followed by MeOH and the glycosides were eluted with MeOH. The eluate was concentrated to less than 1 ml and made up to a 5-ml volume with water. This glucoside solution and the above solution containing aglycones were used for HPLC analysis.

## 2.3. HPLC analysis

HPLC analysis was performed with a Shimadzu LC-10A system liquid chromatograph (Shimadzu Corporation, Japan) equipped with two LC-10 AD pumps with a gradient programmer, an LC-10A UV-Vis spectrophotometer, a CTO-10A column oven and a Chromatopac C-R6A (an integrator). A reversed-phase  $C_{18}$  column (TSK-gel ODS 80Ts, 5  $\mu$ m, 150×4.6 mm I.D., Tosoh Corporation, Japan) was used. The sample was injected by a sample loop valve fitted with a 10- $\mu$ l loop (Rheodyne 7125 injection valve). The separation was performed at 30°C by gradient elution using solvent system I:  $H_2O$ -MeOH-AcOH- $Et_3N$  (A=97:3:0.3:0.3, v/v, pH 4.6; B=20:80:0.3:0.3, v/v) and solvent system II: 1 mM phosphoric acid (pH 3.1)-MeOH (A=97:3, v/v; B=20:80, v/v). The gradient elution was carried out in both solvent systems from A to 9% B in A in 2 min (linear), holding at 9% B in A for 18 min, continuing from 9 to 22% B in A in 30 min (linear), from 22 to 61% B in A in 30 min (linear), from 61 to 100% B in A in 20 min (linear) and finally holding at 100% B for 20 min. The flow-rate was 0.6 ml/min and eluate was monitored at 280 nm.

## 3. Results and discussion

The separations of glucosides and aglycones by HPLC are shown in Figs. 2 and 3. These authentic compounds were separated clearly. In the case of separation with solvent system I (Fig. 2), glucosides were eluted earlier than the aglycones and they approached each other. However, we could obtain a good separation of the glucosides under this condition. The column length was longer (250 mm) and, consequently, the separation was better. Isocratic

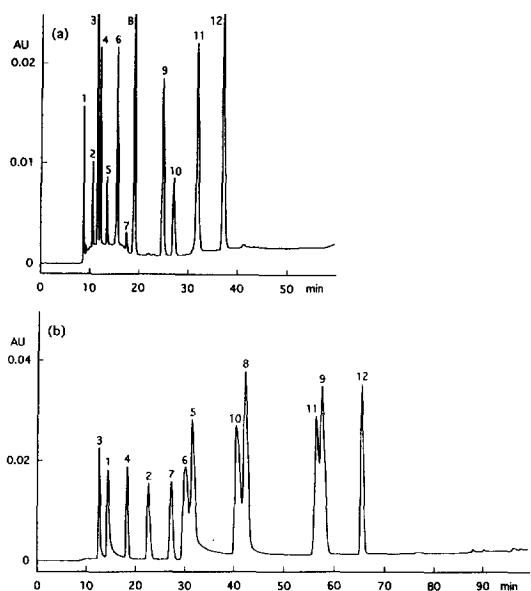


Fig. 2. Separation of glucosides and their aglycones by HPLC with solvent system I (pH 4.6). (a) Glucosides; (b) aglycones. Column: TSK-gel ODS 80Ts (150×4.6 mm I.D., 5  $\mu$ m). Detection: absorbance at 280 nm. Injection volume: 2  $\mu$ l (amounts of compounds, 0.2–2.2 nmol). Flow-rate: 0.6 ml/min. Peaks as in Tables 1 and 2. Gradient elution: solvent system I; H<sub>2</sub>O–MeOH–AcOH–Et<sub>3</sub>N (A=97:3:0.3:0.3, v/v, B=20:80:0.3:0.3, v/v); from A to 9% B in A in 2 min (linear), holding at 9% B in A for 18 min, continuing from 9% to 22% B in A in 30 min (linear), from 22% to 61% B in A in 30 min (linear), from 61% to 100% B in A in 20 min (linear), and finally holding at 100% B for 20 min.

elutions with solvent A (about 3% MeOH) or 9% B in A (about 10% MeOH) did not give good results in the separation of C<sub>6</sub>–C<sub>1</sub> glucosides. The gradient elution from A to 9% B in A in 2 min (linear) at 30°C is necessary to obtain a good separation of C<sub>6</sub>–C<sub>1</sub> glucosides using a TSK-gel ODS 80Ts column. Although solvent system I at pH 4.6 gave good separation of glucosides (Fig. 2), changing the pH of the solvent to 3.1 (as solvent system II) delayed the elution of compounds having a carboxyl group (Fig. 3), and consequently there was incomplete separation of glucosides of C<sub>6</sub>–C<sub>1</sub> compounds. However, separation of aglycones by solvent system II was better than that by solvent system I. The tailings of acid peaks were not significant in solvent system II. The retention times of the compounds are summarized in Table 2.

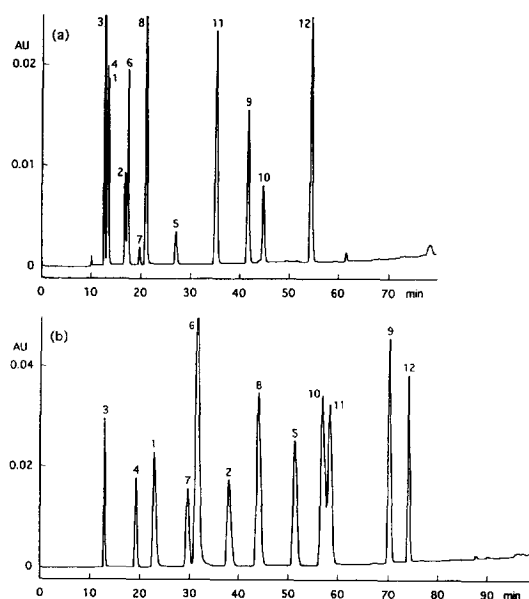


Fig. 3. Separation of glucosides and their aglycones by HPLC with solvent system II (pH 3.1). (a) Glucosides; (b) aglycones. Conditions are the same as in Fig. 2 except that solvent system II, 1 mM phosphoric acid–MeOH (A=97:3, v/v, B=20:80, v/v) was used.

There is a problem of overlapping in the retention times of glucosides and aglycones. Vanillic acid- $\beta$ -D-glucoside and 3,4-dihydroxybenzoic acid, as well as caffeic acid- $\beta$ -D-glucoside and vanillyl alcohol, were not separated in the solvent at pH 4.6. With the solvent system II (pH 3.1), however, these compounds were separated from each other because the acids eluted later on. Furthermore, there are incomplete separations of C<sub>6</sub>–C<sub>1</sub> alcohols from glucosides. Changing the pH of the solvent did not solve the problem, but the use of the starting solvent (3% MeOH, pH 4.6 and 3.1) made complete separations of 4-hydroxybenzyl alcohol- $\beta$ -D-glucoside ( $t_R$ , 18.2 min at pH 4.6 and 20.9 min at pH 3.1) from 3,4-dihydroxybenzyl alcohol ( $t_R$ , 16.0 min at pH 4.6 and 17.2 min at pH 3.1), and vanillyl alcohol- $\beta$ -D-glucoside ( $t_R$ , 40.6 min at pH 4.6 and 46.8 min at pH 3.1) from 4-hydroxybenzyl alcohol ( $t_R$ , 28.0 min at pH 4.6 and 30.6 min at pH 3.1) possible. Thus, it is possible to separate 24 compounds completely by the combination of two different solvent systems. In addition, it is better to extract aglycones with ether

Table 2  
Retention times of authentic compounds

Solvent system I (pH 4.6)										Solvent system II (pH 3.1)									
Aglycone <sup>a</sup>					Glucoside <sup>a</sup>					Aglycone <sup>a</sup>					Glucoside <sup>a</sup>				
<i>t<sub>R</sub></i> (min)	R <sub>1</sub>	R <sub>2</sub>	No. <sup>b</sup>	<i>t<sub>R</sub></i> (min)	R <sub>1</sub>	R <sub>2</sub>	No. <sup>b</sup>	<i>t<sub>R</sub></i> (min)	R <sub>1</sub>	R <sub>2</sub>	No. <sup>b</sup>	<i>t<sub>R</sub></i> (min)	R <sub>1</sub>	R <sub>2</sub>	No. <sup>b</sup>				
9.0	OH	COOH	[1]																
10.9	H	COOH	[2]																
11.8	OH	CH <sub>2</sub> OH	[3]					12.7	OH	CH <sub>2</sub> OH	[3]								
12.4 <sup>c</sup>	H	CH <sub>2</sub> OH	[4]	12.5	OH	CH <sub>2</sub> OH	(3)	13.3	H	CH <sub>2</sub> OH	[4]	13.0	OH	CH <sub>2</sub> OH	(3)				
13.8 <sup>d</sup>	OCH <sub>3</sub>	COOH	[5]	14.2	OH	COOH	(1)	13.5	OH	COOH	[1]								
15.9	OH	CHO	[6]					16.7	H	COOH	[2]								
17.8 <sup>e</sup>	OCH <sub>3</sub>	CH <sub>2</sub> OH	[7]	18.2	H	CH <sub>2</sub> OH	(4)	17.3	OH	CHO	[6]								
19.4	H	CHO	[8]					19.7	OCH <sub>3</sub>	CH <sub>2</sub> OH	[7]	19.1	H	CH <sub>2</sub> OH	(4)				
								21.0	H	CHO	[8]								
25.2	H	CH=CHCOOH	[9]	22.6	H	COOH	(2)					22.5	OH	COOH	(1)				
27.4 <sup>f</sup>	OH	CH=CHCOOH	[10]	27.0	OCH <sub>3</sub>	CH <sub>2</sub> OH	(7)	27.0	OCH <sub>3</sub>	COOH	[5]	28.9	OCH <sub>3</sub>	CH <sub>2</sub> OH	(7)				
				29.4	OH	CHO	(6)					30.9	OH	CHO	(6)				
32.2	OCH <sub>3</sub>	CHO	[11]	31.4	OCH <sub>3</sub>	COOH	(5)												
37.5	OCH <sub>3</sub>	CH=CHCOOH	[12]					35.2	OCH <sub>3</sub>	CHO	[11]	37.4	H	COOH	(2)				
				39.9	OH	CH=CHCOOH	(10)	41.8	H	CH=CHCOOH	[9]	43.6	H	CHO	(8)				
				42.0	H	CHO	(8)	44.9	OH	CH=CHCOOH	[10]	50.7	OCH <sub>3</sub>	COOH	(5)				
				55.9	OCH <sub>3</sub>	CHO	(11)	54.4	OCH <sub>3</sub>	CH=CHCOOH	[12]	56.2	OH	CH=CHCOOH	(10)				
				56.9	H	CH=CHCOOH	(9)					58.0	OCH <sub>3</sub>	CHO	(11)				
				64.7	OCH <sub>3</sub>	CH=CHCOOH	(12)					69.9	H	CH=CHCOOH	(9)				
												74.0	OCH <sub>3</sub>	CH=CHCOOH	(12)				

<sup>a</sup> Names are indicated in Table 1.

<sup>b</sup> Numbers correspond to peak numbers in Figs. 2 and 3.

<sup>c,d,e,f</sup> See Section 3 in the text.

prior to the analysis by HPLC, especially in the case of analysis of glucosides in plant materials (see Section 2). This helps to eliminate complex analysis patterns.

These methods were applied to the analyses of compounds in the green beans of *Vanilla planifolia*. Vanilla extracts were pretreated by extracting with ether and adsorbing to Amberlite XAD-2 column. The resulting samples were analyzed by HPLC with solvent system I. The separation patterns are shown in Fig. 4. In the ether extracts, 4-hydroxybenzaldehyde and vanillin were identified by comparing the retention times with those of the authentic compounds. In the analysis of glucoside fractions, a large amount of glucovanillin and glucoside of 4-hydroxybenzaldehyde were detected. Extraction of aglycones with ether seems to have been effective. More than four compounds were also detected from vanilla green beans by other researchers [11–14]. The amount of the four compounds, 4-hydroxybenzaldehyde, vanillin, 4-hydroxybenzaldehyde- $\beta$ -D-glucoside and glucovanillin in the 6 months old

vanilla green bean were determined as 11.1, 27.6, 212.4 and 1270 mg/100 g fresh weight, respectively.

Consequently, our HPLC analytical method is an appropriate one for performing our study on the biosynthesis of vanillin and will be applied to the measurement of plant components.

### Acknowledgments

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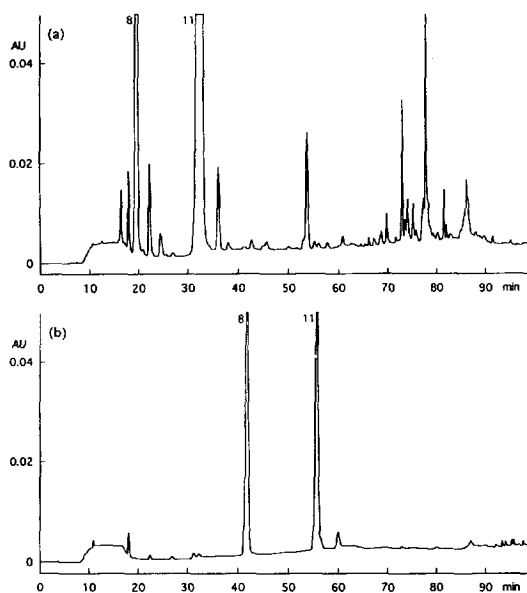


Fig. 4. HPLC analysis of the compounds extracted from *Vanilla planifolia* green bean. Conditions are as in Fig. 2. Gradient elution was done using solvent system I. Samples: vanilla extracts; (a) fraction adsorbed to Amberlite XAD-2 column; (b) ether fraction. Peaks as in Tables 1 and 2.

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