Analysis of Glycosidically Bound Aroma Precursors in Tea Leaves. 1. Qualitative and Quantitative Analyses of Glycosides with Aglycons as Aroma Compounds

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Twenty-six synthetic glycosides constituting aglycons of the main tea aroma compounds ((*Z*)-3-hexenol, benzyl alcohol, 2-phenylethanol, methyl salicylate, geraniol, linalool, and four isomers of linalool oxides) were synthesized in our laboratory as authentic compounds. Those compounds were used to carry out a direct qualitative and quantitative determination of the glycosides as aroma precursors in different tea cultivars by capillary gas chromatographic−mass spectrometric (GC−MS) analyses after trifluoroacetyl conversion of the tea glycosidic fractions. Eleven *β*-D-glucopyranosides, 10 *β*-primeverosides (6-*O*-*β*-D-xylopyranosyl-*β*-D-glucopyranoside) with aglycons as the above alcohols, and geranyl *β*-vicianoside (6-*O*-α-L-arabinopyranosyl-*β*-D-glucopyranoside) were identified (tentatively identified in the case of methyl salicylate *β*-primeveroside) in fresh tea leaves and quantified on the basis of calibration curves that had been established by using the synthetic compounds. Primeverosides were more abundant than glucosides in each cultivar we investigated for making green tea, oolong tea, and black tea. Separation of the diastereoisomers of linalool and four isomers of linalool oxides by GC analyses is also discussed.

Keywords: *Tea aroma formation; aroma precursor; primeveroside; glucoside; glycoside of linalool oxide*

INTRODUCTION

It is well-known that most of the alcoholic tea aroma compounds are mainly present as glycosides in fresh tea leaves and are released by endogenous glycosidases during the manufacturing process of various tea products, especially of semi-fermented tea (oolong tea) and fermented tea (black tea). Since Yano et al. (1991) first isolated benzyl *β*-D-glucopyranoside from fresh leaves of a green tea cultivar, several glycosides as tea aroma precursors have been isolated from various fresh tea leaves which are used to produce green tea, oolong tea, and black tea. These glycosides have been isolated and identified as *β*-D-glucopyranoside (Glc), 6-*O*-*β*-D-xylopyranosyl-*β*-D-glucopyranoside (primeveroside, Prim), 6-*O*-α-L-arabinopyranosyl-*β*-D-glucopyranoside (vicianoside, Vic) and 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranoside (acuminoside, Acu) with monoterpene alcohol aglycons of geraniol, linalool, and linalool oxides (LOs), with aromatic alcohol aglycons of benzyl alcohol, 2-phenylethanol, with phenol aglycone of methyl salicylate, and with the aliphatic alcohol aglycone of (*Z*)-3-hexenol (Kobayashi et al., 1994; Guo et al., 1993, 1994; Moon et al., 1994, 1996; Nishikitani et al., 1996, 1999).

Several studies have reported the constituents and amounts of glycosidically bound aroma compounds in tea leaves by using enzymatic hydrolysis (Morita et al., 1994; Ogawa et al., 1995; Sakata et al., 1995). However, there are no quantitative data for the individual components of these glycosidically bound aroma precursors. Gas chromatographic−mass spectrometric (GC−MS) analyses of glycosides via trifluoroacetyl conversion has recently been proven to be an effective method for directly quantifying the glycosides in grape, mango, and passion fruit (Voirin et al., 1990, 1992a, 1992b; Lopez-Tamames et al., 1997; Sakho et al.,1997; Chassagne et al., 1997).

We have recently established a method to synthesize the main diglycosides of tea aroma (Matsumura et al., 1997) and synthesized several of the main glycosides (shown in Figure 1) that have been positively identified as, or are thought to be, tea aroma precursors. The trifluoroacetyl (TFA) derivatives of these authentic samples were compared to those of the glycosides in tea leaves, and the changes in the amounts of 18 glycosides during the black tea manufacturing process were presented (Kobayashi et al., 1999). In the present report, the identification of these glycosides, including some new glycosides, is discussed in more detail, and the amounts of each glycoside in various tea cultivars are calculated based on the equations of each calibration curve by GC−MS analyses.

MATERIALS AND METHODS

Reagents and Reference Samples. Analytical-reagent-grade solvents were used. Amberlite XAD-2 resin and the trifluoroacetylating reagent [*N*-methyl bis(trifluoroacetamide), MBTFA] were purchased from Organo (Japan) and Pierce (Rockford, IL), respectively. Phenyl *β*-D-glucopyranoside was purchased from Tokyo Kasei Kogyo Co. (Japan). The (*Z*)-3-hexenyl, benzyl, 2-phenylethyl, geranyl and (*RS*)-linalyl *β*-D-glucopyranosides and *β*-primeverosides, and geranyl *β*-vicianoside were each synthesized according to the method of

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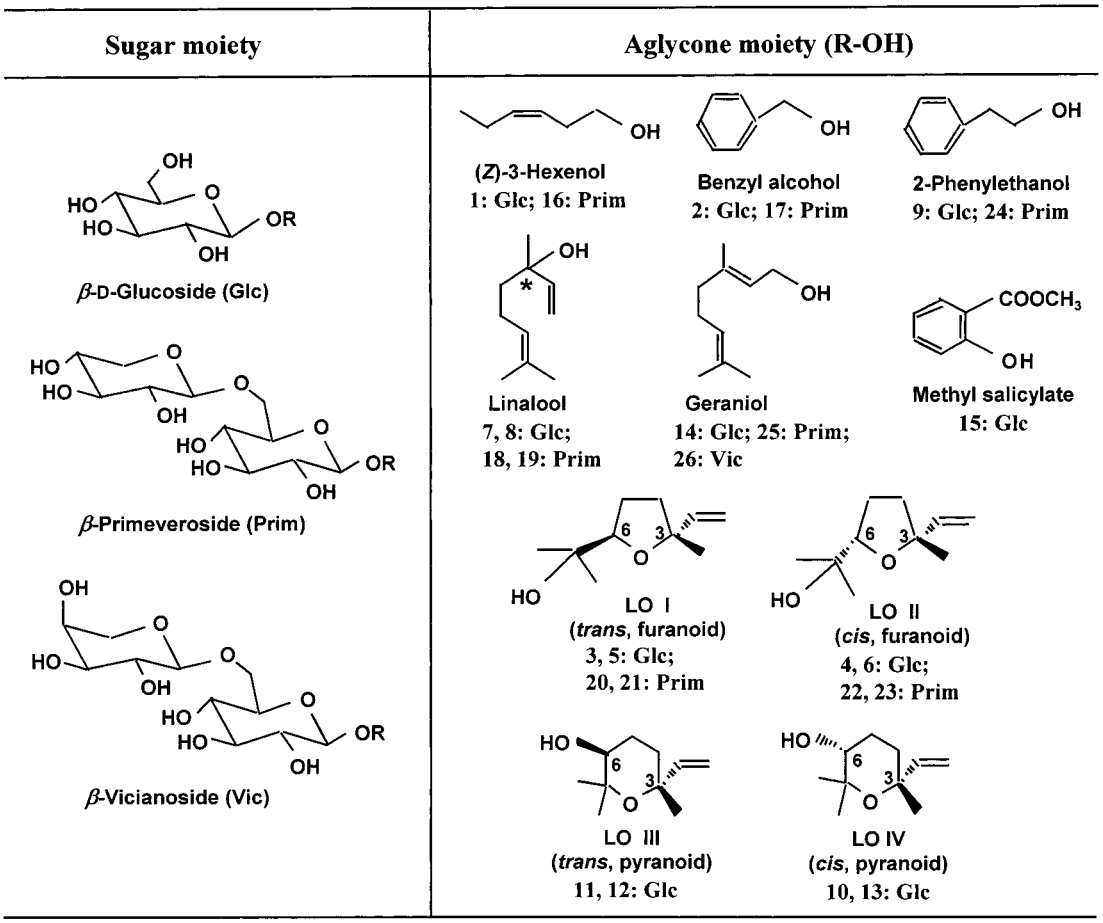

Figure 1. Structures of the synthesized glycosides as tea aroma precursors. Numbers in the aglycone moiety section correspond to numbers in tables and figures hereafter.

Matsumura et al. (1997). The same method was used to newly synthesize the β-D-glucopyranosides of four isomers of linalool oxide (LO I, *trans*-furanoid; LO II, *cis*-furanoid; LO III. *trans*-pyranoid; and LO IV, *cis*-pyranoid), and β-primeverosides of LO I and LO II (details will be reported elsewhere). Methyl salicylate β-D-glucopyranoside was synthesized under the experimental conditions described by Mulkens and Kapetanidis (1988).

Samples of the Tea Leaves. Fresh leaves of *Camellia sinensis* var. *sinensis* cv. Yabukita (for making green tea) and cv. Benihomare (a hybrid of var. *sinensis* and var. *assamica*, for making black tea) were harvested at the National Research Institute of Vegetables, Ornamental Plants and Tea (Kanaya, Shizuoka, Japan) in April and May 1998, respectively. After being plucked, the fresh leaves were immediately frozen by adding liquid nitrogen, and then lyophilized to dryness. Fresh leaves of cv. Chin-Shin-Oolong (for making oolong tea) were plucked and immediately dried by heating (parching at 230 °C for 4−5 min, and then drying at 88−98 °C) at the Taiwan Tea Experiment Station in April 1998.

Preparation of the Glycosidic Fractions. Glycosidic fractions from the three samples of dried fresh tea leaves were each prepared by using XAD-2 column chromatography (Günata et al., 1985). A sample of 20 g of dried tea leaves was crushed with a Willey-style crusher, 1 mL of an aqueous solution of phenyl β-D-glucopyranoside (1.5 mg/mL) was added as an internal standard, and then the sample was extracted twice with boiling water (280 mL and 120 mL) for 10 min each. The extract was passed through a nylon mesh filter and cooled to room temperature in an ice water mixture. Then 10 g of Polyclar AT (General Anilin & Film Corp., New York) was added, and the solution was stirred for 20 min to absorb the polyphenols before the solid matter was removed by a suction filter with a thin silica gel layer. This operation was repeated by adding 5 g of Polyclar AT. The filtrate was concentrated to 70 mL under reduced pressure at 40 °C and then centrifuged at 13 500 rpm (20 000*g* × 20 min × 2). To the supernatant was added 280 mL of methanol to precipitate the protein, and then the supernatent was filtered. The filtrate was concentrated under reduced pressure to remove the methanol completely. The resulting aqueous residue was subjected to chromatography with an Amberlite XAD-2 adsorbent in a 28 × 4 cm (i.d.) glass column. After successively washing the column with 2000 mL of water and 800 mL of pentane−ether (1:1), the glycosidic fraction was obtained by eluting with 1000 mL of methanol. The eluate was concentrated to dryness and treated as the crude glycosidic fraction.

Trifluoroacetylation of the Synthetic and Natural Glycosides. *N*-methyl bis(trifluoroacetamide) (MBTFA) was used as the TFA reagent which has been reported by Sullivan and Schewe (1977) to cleanly produce the trifluoroacetates of some mono-, di-, tri- and tetrasaccharides with high volatility. To a mixture of 10 or 16 synthetic glycosides (about 0.1 mg of each compound) in a small screw-capped vial, 20 μL of anhydrous pyridine and 20 μL of MBTFA were added under nitrogen. The vial was tightly closed, and the contents were stirred. The vial was heated at 60 °C for 30 min and then allowed to cool to room temperature before being subjected to GC−MS analyses.

Each sample (8 mg) of the natural glycosidic fractions was treated in a similar manner with 25 μL of anhydrous pyridine and 30 μL of MBTFA, and each was heated for 50 min.

GC−MS Analyses of TFA Derivatives of the Glycosides. A Hewlett-Packard 5890 Series II gas chromatograph equipped with a Hewlett-Packard 5972 Series mass selective detector was used for the GC−MS analyses. The TFA derivatives of the glycosides prepared as described above were analyzed in a DB-5 ((5% phenyl)-methylpolysiloxane) fused-silica capillary column (J&W, 60 m × 0.25 mm i.d., 0.25 μm bonded phase) with a weakly polar stationary phase, and in a

Table 1. Retention Indices of the TFA Derivatives of Authentic Glycosides in the DB-5 Column and HP-50+ Column

		RRT[a]				RRT[a]	
no.	glucoside	DB-5	HP-50+	no.	disaccharide	DB-5	HP-50+
1	(*Z*)-3-hexenyl-Glc	0.896	0.873	16	(*Z*)-3-hexenyl-Prim	1.685	1.513
2	benzyl-Glc	1.126	1.123	17	benzyl-Prim	1.877	1.708
3	(3*S*,6*S*)-LO I-Glc	1.167	1.055	18	(*R*)-linalyl-Prim	1.870	1.619
4	(3*R*,6*S*)-LO II-Glc	1.174	1.064	19	(*S*)-linalyl-Prim	1.896	1.645
5	(3*R*,6*R*)-LO I-Glc	1.185	1.064	20	(3*R*,6*R*)-LO I-Prim	1.896	1.648
6	(3*S*,6*R*)-LO II-Glc	1.193	1.074	21	(3*S*,6*S*)-LO I-Prim	1.896	1.650
7	(*R*)-linalyl-Glc	1.191	1.053	22	(3*R*,6*S*)-LO-II-Prim	1.896	1.653
8	(*S*)-linalyl-Glc	1.203	1.069	23	(3*S*,6*R*)-LO-II-Prim	1.908	1.665
9	2-phenylethyl-Glc	1.272	1.259	24	2-phenylethyl-Prim	1.978	1.782
10	(3*R*,6*R*)-LO-IV-Glc	1.284	1.169	25	geranyl-Prim	2.039	1.798
11	(3*S*,6*R*)-LO-III-Glc	1.298	1.188	26	geranyl-Vic	2.061	1.898
12	(3*R*,6*S*)-LO-III-Glc	1.316	1.194				
13	(3*S*,6*S*)-LO-IV-Glc	1.319	1.188				
14	geranyl-Glc	1.343	1.227				
15	methyl salicylate-Glc	1.455	1.465				

[a] RRT, relative retention time ratio to phenyl β-D-glucoside.

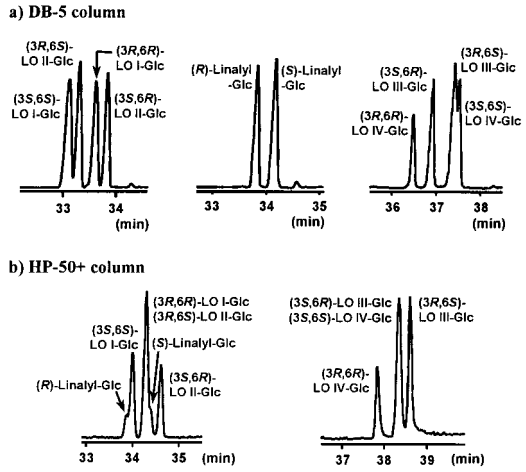

Figure 2. Gas chromatograms of TFA derivatives of the glucosides of linalool and LOs in the DB-5 column and HP-50+ column.

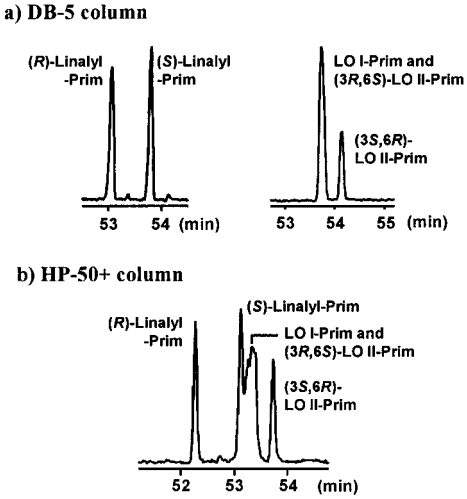

Figure 3. Gas chromatograms of TFA derivatives of the primeverosides of linalool, LO I, and LO II in the DB-5 column and HP-50+ column.

HP-50+ ((50% phenyl)-methylpolysiloxane) fused-silica capillary column (Hewlett-Packard, 30 m × 0.25 mm i.d., 0.25 μm bonded phase) with a medium-polar stationary phase, respectively. A 0.6-μL aliquot of the synthetic glycoside derivatives or 1.5 μL of the natural glycoside derivatives was injected at a 20:1 split ratio into an injector held at 280 °C. The column temperature was held at 130 °C for 2 min and then raised to 280 °C at the rate of 2 °C/min for the DB-5 column, and was held at 80 °C for 2 min and then raised to 280 °C at the rate of 2 °C/min for the HP-50+ column. Helium was used as the carrier gas at a flow rate of 1.0 mL/min.

Electron impact (EI) spectra were recorded; the temperature of the ion source was 180 °C. Mass spectra were scanned at 70 eV in an *m*/*z* range from 30 to 700 mass units.

Identification of the Natural Glycosides in Tea Leaves. Phenyl β-D-glucopyranoside was used as an internal standard (IS). The natural glycosides were identified by comparing the relative retention time ratio (RRT) to that of the IS in DB-5 or HP-50+ column, and by comparing the MS data with those of the authentic synthesized glycosides.

Preparation of the Calibration Curves for the Glycosides. A series of mixtures of the authentic synthesized glucosides and primeverosides in different amount ratios to the IS (0.01, 0.2, 1, 5, and 50) were trifluoroacetylated and analyzed by GC−MS. To avoid coelution in the GC analysis of the TFA derivatives of the glycosides, two synthetic glycoside mixtures with different constituents were prepared for each concentrate. The experiment was performed twice for each concentrate.

Quantification of the Natural Glycosides in the Different Tea Leaf Samples. The content of natural glycosides in each fresh tea leaf sample was determined by using the calibration curves already established and is expressed by the average of three repeated processes from extraction to the GC−MS analysis.

RESULTS AND DISCUSSION

Gas Chromatographic Behavior of TFA Derivatives of the Authentic Synthesized Glycosides. The mixture of synthetic glycosides (fifteen glucosides and eleven disaccharides, mainly primeverosides) was trifluoroacetylated and subjected to GC−MS analyses with weakly polar column (DB-5) and medium-polar column (HP-50+), respectively. The relative retention time (RRT) ratios to IS are summarized in Table 1. Almost all of the glycosides studied could be well-separated, except for a few instances, as shown in Table 1. RRT values for the glucosides of benzyl alcohol, 2-phenyl ethanol, geraniol, and (*R*,*S*)-linalool in the DB-5 column matched the results reported by Voirin et al. (1992a) who used a CP-Sil 8 CB column (Chrompack, almost the same as the DB-5 column).

With regard to the diastereoisomers, as shown in Figure 2, the β-D-glucopyranosides of (*R*,*S*)-linalool and the eight stereoisomers of linalool oxides were each well-separated in the DB-5 column. The elution order was determined by using individual synthetic glucoside with known absolute configurations. However, the peak of

Table 2. Mass Spectra of TFA Derivatives of the Synthetic β-D-Glucopyranosides

		EI−MS: characteristic fragment ions (*m*/*z*)	
no.	glucoside	aglycone moiety	sugar moiety
1	(*Z*)-3-hexenyl-Glc	55(100), 69(87), 83(55), 82(56), 81(23), 97(11)	319(29), 193(7.0), 177(7.0), 205(4.7), 265(1.0)
3	(3*S*,6*S*)-LO I-Glc	111(100), 93(60), 69(49), 153(31), 71(25), 81(23), 83(13), 94(6.0)	319(43), 193(6.4), 177(4.7), 205(3.1), 265(2.7), 547(0.6)
4	(3*R*,6*S*)-LO II-Glc	111(100), 93(59), 69(51), 153(31), 71(25), 81(23), 83(12), 94(6.1)	319(49), 193(6.9), 177(4.9), 205(3.7), 265(2.9), 547(0.5)
5	(3*R*,6*R*)-LO I-Glc	111(100), 93(58), 69(49), 153(26), 71(23), 81(22), 83(12), 94(5.9)	319(42), 193(5.9), 177(4.6), 205(3.1), 265(2.4), 547(0.5)
6	(3*S*,6*R*)-LO II-Glc	111(100), 93(61), 69(50), 153(24), 71(22), 81(23), 83(13), 94(6.0)	319(46), 193(6.2), 177(4.9), 205(3.5), 265(2.5), 547(0.5)
10	(3*R*,6*R*)-LO IV-Glc	94(100), 68(93), 81(63), 93(51), 71(31), 95(23), 111(22), 137(19), 153(17)	319(31), 177(10), 193(8.8), 205(6.0), 265(1.7)
11	(3*S*,6*R*)-LO III-Glc	68(100), 94(95), 81(67), 93(48), 71(31), 95(21), 111(21), 137(21), 153(16)	319(21), 177(9.3), 193(8.5), 205(5.6)
12	(3*R*,6*S*)-LO III-Glc	94(100), 68(99), 81(69), 93(55), 71(28), 95((23), 111(25), 137(26), 153(17)	319(26), 177(11), 193(7.4), 205(5.0)
13	(3*S*,6*S*)-LO IV-Glc	94(100), 68(91), 81(61), 93(50), 71(29), 95(22), 111(21), 137(18), 153(17)	319(31), 177(10), 193(8.2), 205(6.2), 265(1.2)
15	methyl salicylate-Glc	152(100), 81(12), 92(19), 120(78), 121(36)	319(7.1), 177(9.3), 193(4.2), 205(3.6)

Table 3. Mass Spectra of TFA Derivatives of the Synthetic β-Primeverosides and β-Vicianosides

		EI−MS: characteristic fragment ions (*m*/*z*)	
no.	disaccharide	aglycone moiety	sugar moiety
16	(*Z*)-3-hexenyl-Prim	83(100), 55(97), 82(73), 81(22), 69(72), 67(26), 97 (19)	193(65), 165(5.5), 265(1.5), 278(1.4), 177(1.9), 307(0.3)
17	benzyl-Prim	91(100), 92(12), 107(15), 108(5.5)	193(24), 165(1.6), 265(1.0), 278(0.8), 177(0.6), 307(0.2), 319(0.1), 421(0.1)
18	(*R*)-linalyl-Prim	69(100), 81(67), 93(61), 80(35), 136(32), 137(23), 121(17), 153(7.7)	193(66), 165(5.3), 265(3.2), 278(2.3), 177(1.8), 307(1.6), 421(1.0), 319(0.8)
19	(*S*)-linalyl-Prim	69(100), 81(68), 93(61), 80(37), 136(21), 137(22), 121(17), 153(8.0)	193(64), 165(5.0), 265(3.2), 278(2.2), 177(2.0), 307(1.7), 421(1.1), 319(0.7)
20	(3*R*,6*R*)-LO I-Prim	111(100), 93(45), 69(31), 153(54), 71(23), 81(19), 83(7.8), 94(4.8)	193(63), 165(4.9), 265(2.1), 278(1.7), 177(1.5), 307(2.3), 421(1.4), 319(0.6)
21	(3*S*,6*S*)-LO I-Prim	111(100), 93(45), 69(32), 153(61), 71(25), 81(20), 83(8.3), 94(4.9)	193(60), 165(4.5), 265(2.1), 278(1.5), 177(1.7), 307(2.1), 421(1.2), 319(0.6)
22	(3*R*,6*S*)-LO II-Prim	111(100), 93(47), 69(36), 153(60), 71(26), 81(21), 83(8.3), 94(5.0)	193(69), 165(5.3), 265(2.5), 278(1.7), 177(1.9), 307(2.7), 421(1.7), 319(0.8)
23	(3*S*,6*R*)-LO II-Prim	111(100), 93(45), 69(35), 153(54), 71(23), 81(20), 83(8.5), 94(4.7)	193(70), 165(5.1), 265(2.4), 278(1.6), 177(1.7), 307(2.7), 421(1.6), 319(0.7)
24	2-phenylethyl-Prim	105(100), 104(45), 91(16), 106(16)	193(33), 165(2.7), 265(1.0), 278(0.8), 177(0.7), 307(0.3), 279(0.6), 319(0.2)
25	geranyl-Prim	69(100), 81(26), 123(11), 68(14), 93(9.6), 95(8.5)	193(29), 165(2.1), 265(0.9), 278(0.6), 177(0.8), 307(0.8), 319(0.2), 421(0.4)
26	geranyl-Vic	69(100), 81(30), 123(12), 68(16), 93(9.5), 95(11)	193(33), 165(2.5), 265(0.9), 278(0.6), 177(0.9), 307(0.4), 319(0.2), 421(1.4)

Table 4. Equations for the Calibration Curves of Glycosides for GC−MS Analyses in the Two Different Columns[a]

glycoside	DB-5 column	HP-50+ column
(*Z*)-3-hexenyl-Glc	$y = 1.62x - 0.15$ $R^2 = 0.9935$	$y = 1.63x - 0.24$ $R^2 = 0.9930$
benzyl-Glc	$y = 1.44x - 0.08$ $R^2 = 0.9995$	$y = 1.29x - 0.03$ $R^2 = 0.9662$
linalyl-Glc	$y = 0.98x - 0.07$ $R^2 = 0.9996$	$y = 1.13x - 0.11$ $R^2 = 0.9969$
LO I&II-Glc	$y = 1.07x - 0.05$ $R^2 = 0.9971$	$y = 1.31x - 0.07$ $R^2 = 0.9905$
2-phenylethyl-Glc	$y = 1.32x - 0.03$ $R^2 = 0.9976$	$y = 1.49x - 0.10$ $R^2 = 0.9967$
methyl salicylate-Glc	$y = 0.66x - 0.02$ $R^2 = 0.9988$	$y = 0.66x - 0.03$ $R^2 = 0.9983$
LO III&IV-Glc	$y = 1.11x - 0.05$ $R^2 = 0.9971$	$y = 0.74x + 0.08$ $R^2 = 0.9928$
geranyl-Glc	$y = 1.29x - 0.13$ $R^2 = 0.9991$	$y = 1.24x - 0.09$ $R^2 = 0.9953$
(*Z*)-3-hexenyl-Prim	$y = 1.43x + 0.01$ $R^2 = 0.9935$	$y = 1.49x - 0.09$ $R^2 = 0.9962$
benzyl-Prim	$y = 1.29x - 0.13$ $R^2 = 0.9978$	$y = 1.00x + 0.03$ $R^2 = 0.9988$
linalyl-Prim	$y = 0.53x - 0.08$ $R^2 = 0.9963$	$y = 1.11x - 0.15$ $R^2 = 0.9954$
LO I&II-Prim	$y = 0.97x - 0.05$ $R^2 = 0.9975$	$y = 1.33x - 0.29$ $R^2 = 0.9964$
2-phenylethyl-Prim	$y = 1.05x - 0.01$ $R^2 = 0.9966$	$y = 0.99x - 0.02$ $R^2 = 0.9988$
geranyl-Prim	$y = 1.30x - 0.19$ $R^2 = 0.9921$	$y = 1.31x - 0.22$ $R^2 = 0.9926$
geranyl-Vic	$y = 0.78x - 0.11$ $R^2 = 0.9914$	$y = 0.78x - 0.16$ $R^2 = 0.9907$

[a] *y*: Peak area ratio to IS in the total ion gas chromatogram detected by EI mass spectrometry. *x*: Actual content ratio to IS.

(*R*)-linalyl glucoside was eluted with almost the same retention time as that of (3*S*,6*R*)-LO II glucoside. Although separation in the HP-50+ column was slightly worse than that in the DB-5 column (Figure 2), the glucosides of LOs could be detected without coelution with other contaminants, this being described in the next section. With respect to the primeverosides, the (*R*,*S*)-linalyl *β*-primeverosides were well-resolved in both the DB-5 column and the HP-50+ column, and the four stereoisomers of LO I and II were only separated into two peaks, their elution order being shown in Figure 3.

Table 5. Extraction Coefficients of Glycosides on XAD-2 Column Chromatography

compound	starting amount[a] (mg)	mean extraction coefficient (%)	reproducibility[b] (%)
(*Z*)-3-hexenyl-Glc	3.0	100.3	0.7
benzyl-Glc	2.5	99.3	0.8
linalyl-Glc	3.0	97.6	1.3
LO I-Glc	4.0	104.5	1.2
2-phenylethyl-Glc	2.5	104.9	3.2
methyl salicylate-Glc	2.5	96.2	1.0
LO IV-Glc	4.0	96.4	4.6
gernayl-Glc	3.0	94.7	0.9
(*Z*)-3-hexenyl-Prim	3.0	98.4	0.8
benzyl-Prim	2.5	99.5	0.8
linalyl-Prim	3.0	98.4	1.2
LO I-Prim	4.0	102.5	3.5
2-phenylethyl-Prim	2.5	102.3	0.3
geranyl-Prim	3.0	98.2	1.2
geranyl-Vic	3.0	98.6	1.0

[a] Amount (mg) used in the solution of synthesized glycosides mixture for each repetition. [b] Calculated as relative standard deviation ($n = 3$) for the extraction coefficient

Coelution of the (*S*)-linalyl primeveroside and LO I and (3*R*,6*S*)-LO II primeverosides was also apparent from their retention times.

Mass Spectrometric Behavior of TFA Derivatives of the Authentic Synthesized Glycosides. Tables 2 and 3 show EI−MS fragmentation patterns of TFA derivatives of the authentic synthesized glucosides and disaccharides (mainly primeverosides). The glucosides of benzyl alcohol, 2-phenylethanol, geraniol, and linalool reported by Voirin et al. (1992a) have been omitted. The EI−MS data show characteristic fragment ions derived from the aglycone and sugar moiety, respectively. As have been observed for other glycosides of aroma compounds (Voirin et al., 1992a), there was no peak derived from the aglycone moiety present in the mass range of the characteristic fragment ions of the sugar moiety, the fragment ions derived from the aglycone moiety being far more numerous and abundant than those from the sugar moiety.

The fragment ions for the aglycone moiety were quite similar to those from the corresponding free alcohol,

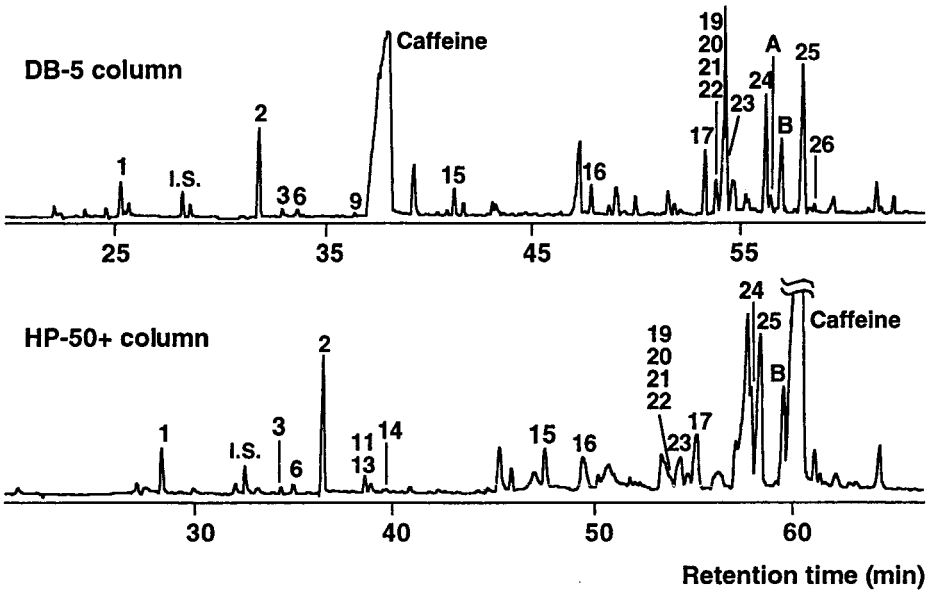

Figure 4. Gas chromatograms of TFA derivatives of the glycosidic extracts from fresh tea leaves of Benihomare.

Table 6. Contents of Glycosides in Different Fresh Tea Leaves

no.	glycoside[a]	content (mg/100 g dried leaves)		
		Yabukita	Chin-Shin-Oolong	Benihomare
1	(*Z*)-3-hexenyl-Glc	10.9 ± 1.0	1.6 ± 0.4	6.6 ± 0.5
2	benzyl-Glc	17.4 ± 1.4	10.0 ± 0.3	23.1 ± 1.1
3	(3*S*,6*S*)-LO I-Glc[b]	0.7 ± 0.1	0.7 ± 0.1	1.7 ± 0.2
4	(3*R*,6*S*)-LO II-Glc[b]	trace	trace	trace
5	(3*R*,6*R*)-LO I-Glc[b]	trace	trace	trace
6	(3*S*,6*R*)-LO II-Glc[b]	0.9 ± 0.1	1.5 ± 0.4	2.6 ± 0.4
9	2-phenylethyl-Glc	0.6 ± 0.0	1.7 ± 0.3	1.3 ± 0.0
11	(3*S*,6*R*)-LO-III-Glc[b]	0.0 ± 0.0	0.1 ± 0.0	1.1 ± 0.2
13	(3*S*,6*S*)-LO-IV-Glc[b]	0.2 ± 0.0	0.2 ± 0.0	3.7 ± 0.7
14	geranyl-Glc[b]	0.4 ± 0.0	1.1 ± 0.3	0.8 ± 0.0
15	methyl salicylate-Glc	4.7 ± 0.4	2.9 ± 0.9	12.7 ± 1.1
	total of glucosides	35.8 ± 3.2	19.7 ± 2.7	53.5 ± 4.2
16	(*Z*)-3-hexenyl-Prim	6.1 ± 0.7	0.6 ± 0.1	6.7 ± 0.8
17	benzyl-Prim	34.4 ± 4.8	10.4 ± 2.2	20.1 ± 1.6
19	(*S*)-linalyl-Prim			
20	(3*R*,6*R*)-LO I-Prim[b]	3.0 ± 0.1	2.0 ± 0.2	5.3 ± 0.8
21	(3*S*,6*S*)-LO I-Prim[b]			
22	(3*R*,6*S*)-LO-II-Prim[b]			
23	(3*S*,6*R*)-LO-II-Prim[b]	7.0 ± 0.2	3.5 ± 0.9	13.4 ± 2.7
24	2-phenylethyl-Prim	8.7 ± 1.3	11.4 ± 2.3	41.1 ± 4.5
A	LO III & IV-disaccharide[c]	2.3 ± 0.3	1.6 ± 0.2	4.4 ± 0.6
B	methyl salicylate-Prim[c]	18.2 ± 3.9	10.0 ± 2.5	45.2 ± 4.2
25	geranyl-Prim	10.5 ± 1.6	9.2 ± 1.5	47.2 ± 7.0
26	geranyl-Vic	1.8 ± 0.0	1.7 ± 0.2	3.6 ± 0.2
	total of disaccharides	92.0 ± 12.9	50.4 ± 10.2	187.0 ± 22.5
	total of glycosides	127.8 ± 16.1	70.1 ± 12.9	240.5 ± 26.7

[a] Positive identification except for methyl salicylate-Prim (tentative identification). [b] Quantitation was based on the GC−MS analyses in HP-50+ column, otherwise, in DB-5 column. [c] Calibration curve of the corresponding glucoside was used for quantitation.

although with a different abundance pattern, which made the identification of the aglycone moiety quite easy.

Regarding the sugar moiety, as reported earlier by Voirin et al. (1992a), the glucose moiety gave the characteristic fragment ions at *m*/*z* 547, 319, 265, 205, 193, and 177, with *m*/*z* 319 being observed most abundantly among them. With the disaccharides, besides the fragment ions at *m*/*z* 319, 265, 193, and 177 derived from the glucopyranose unit, the xylopyranose or arabinopyranose unit gave characteristic fragment ions at *m*/*z* 421, 307, 278, 265, 193, and 165. Like the other disaccharides reported by Voirin et al. (1992a), *m*/*z* 193 was the most abundant fragment ion derived from the sugar moiety. These fragment ions are similar to those reported earlier for the TFA derivatives of glucopyranose and aldopentapyranose (Koenig et al., 1973). Although there were no differences observed between the primeverose and vicianose units, the retention indices were different, enabling us to differentiate between them by comparing the GC retention indices with those of the synthetic glycosides.

Calibration Curves for the Glycosides from GC−MS Analyses. The synthetic glycosides were used to create calibration curves so that the glycosides studied by GC−MS analyses could be established from the total ion chromatograms. The calculated equations for the calibration curves of each glycoside are summarized in Table 4. Almost all of the R^2 values were more than 0.99, except for benzyl glucoside in the HP-50+ column (0.9662), which proves the good linearity of the calibration curves under our experimental conditions. The regression coefficients were quite similar between the glucosides and their corresponding primeverosides. Any differences in the regression coefficients were due to the aglycone moiety more than to the sugar moiety with both of the columns, except in the case of linalyl primeveroside. The low regression coefficient for linalyl primeveroside in the DB-5 column is considered to have been due to the partial decomposition at ca. 242 °C.

Identification of the Natural Glycosides in Tea Leaves. The glycosidic fractions were separated from dried fresh tea leaves of cv. Yabukita, Chin-Shin-Oolong, and Benihomare. After TFA conversion, the reaction mixtures were subjected to GC−MS analyses. Figure 4 shows the total ion gas chromatograms for the TFA derivatives of the glycosidic fraction separated from fresh tea leaves of Benihomare in the DB-5 column and HP-50+ column. A comparison of the retention indices and mass spectra with those of synthetic glycosides enabled eleven glucosides, 10 primeverosides, and one vicianoside to be identified (tentatively identified in the case of methyl salicylate-Prim; Table 6 below). The use of the two different-polarity columns was effective for separation and identification, particularly because the large peak for caffeine in the natural products could be moved from the glucoside region to the disaccharide region by changing the column from DB-5 to HP-50+. The glucosides of LO III, LO IV, and geraniol were observed only in the chromatogram from the HP-50+ column, whereas the disaccharides were better separated in the DB-5 column than in the HP-50+ column. The characteristic fragment ions derived from (*S*)-linalyl primeveroside were found in the coeluted peak of LO I and (3*R*,6*S*)-LO II primeverosides. Besides the known glycosides isolated from various tea leaves that have been reported previously (Kobayashi et al., 1994; Guo et al., 1993, 1994; Moon et al., 1994, 1996; Nishikitani et al., 1996, 1999), the glucosides of LO I, LO II, LO III, LO IV, 2-phenylethanol, geraniol, and methyl salicylate were detected in tea leaves for the first time. Additionally, the disaccharides of LO III and IV were

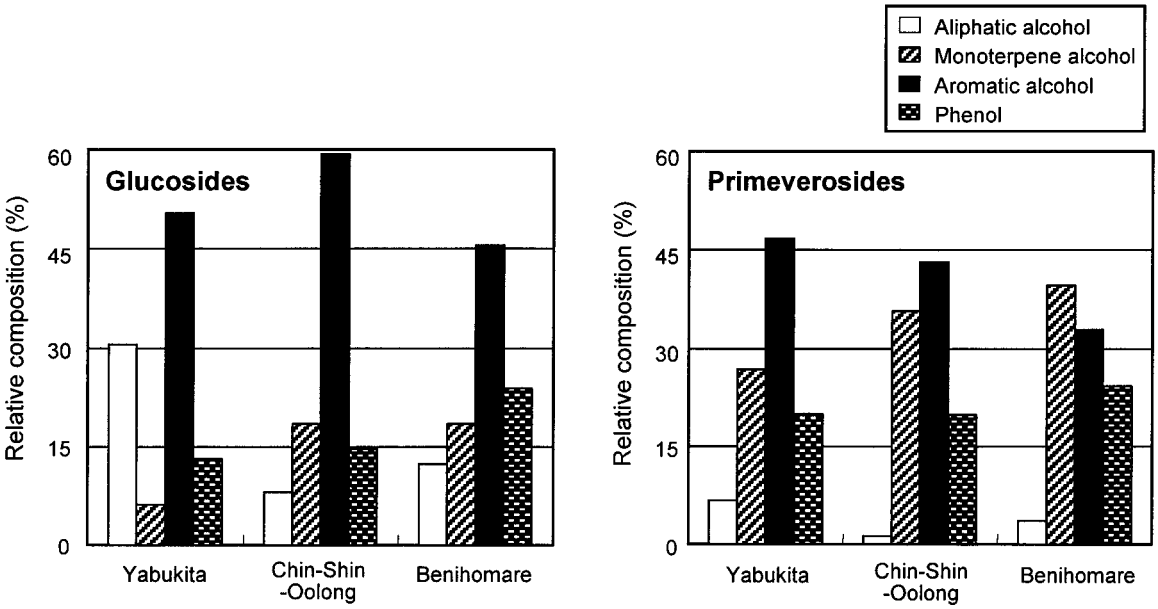

Figure 5. Relative compositions of the glycosides of aliphatic, monoterpene, and aromatic alcohols, and phenol, in the total glucosides and total primeverosides.

also observed. Because we did not have the authentic disaccharides (primeverosides, vicianosides, or acuminosides) of LO III and LO IV, the sugar moiety has not been identified.

Quantification of the Natural Glycosides in the Different Fresh Tea Leaf Samples. Concerning the extraction coefficient of the glycosides on XAD-2 column chromatography, we prepared an aqueous solution of the authentic synthesized glycosides mixture in which 2.5−4.0 mg of each glycoside was contained, and subjected it to XAD-2 column chromatography as described above. As shown in Table 5, the extraction coefficients of all of the glycosides were more than 94.7%. This result infers that the glycosides could be recovered almost completely on XAD-2 column chromatography under our experimental condition.

On the basis of the calibration curves that were established, we determined the contents of the glycosides in the different fresh tea leaves of cv. Yabukita, Chin-Shin-Oolong, and Benihomare for making green tea, oolong tea, and black tea, respectively (Table 6). In each tea leaf sample that we studied, the content of disaccharides was much higher than that of glucosides because of the high content of primeverosides, which shows the high potential of primeverosides as tea aroma precursors.

The relative compositions of the glycosides of aliphatic, monoterpene, and aromatic alcohols, and phenol in the total glucosides and total primeverosides are shown in Figure 5. Both the glucosides and primeverosides of aromatic alcohols were most abundant in each tea leaf sample, except for the primeverosides in Benihomare. However, with respect to primeverosides, monoterpene alcohols shared more weight than that in the total glucosides, especially in the case of Benihomare where the highest percentage could be seen for the primeverosides of monoterpene alcohols. Furthermore, the glycoside (*Z*)-3-hexenol was more abundant in Yabukita and Benihomare than in Chin-Shin-Oolong.

The absolute configuration of the aglycone of LOs shows that 3*S*-configuration LO I and LO II were much more abundant than 3*R*-configuration LO I and LO II in both glucosides and primeverosides. The same pattern was also observed with the glucosides of LO III and LO IV. This result concurs with the enantiomeric composition of each of these compounds in the tea free-aroma concentrates (Wang et al., 1994). This means the chirality of these compounds in tea aroma was mainly influenced by their stereoisomeric composition present as glycosides in fresh tea leaves. Further study on the contribution of the glycosides to tea aroma is proceeding and will be reported in the future.

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