

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/10606863>

Identification of Chlorophylls and Carotenoids in Major Teas by High-Performance Liquid Chromatography with Photodiode Array Detection

ARTICLE in JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · SEPTEMBER 2003

Impact Factor: 2.91 · DOI: 10.1021/jf030158d · Source: PubMed

CITATIONS

29

READS

46

2 AUTHORS:



Yasuyo Suzuki

Aichi Human Service Center

24 PUBLICATIONS 187 CITATIONS

SEE PROFILE



Yuzo Shioi

Ma Chung University

106 PUBLICATIONS 1,773 CITATIONS

SEE PROFILE

Identification of Chlorophylls and Carotenoids in Major Teas by
High-Performance Liquid Chromatography with Photodiode
Array Detection

YASUYO SUZUKI AND YUZO SHIOI*

Department of Biology and Geoscience, Faculty of Science, Shizuoka University,
Shizuoka 422-8529, Japan

The separation and identification of pigments, chlorophylls, and carotenoids of seven teas and fresh leaf of tea (*Camellia sinensis*) by high-performance liquid chromatography (HPLC) are described. HPLC was carried out using a Symmetry C₈ column with a photodiode array detector. Pigments were eluted with a binary gradient of aqueous pyridine solution at a flow rate of 1.0 mL/min at 25 °C. HPLC analyses achieved the separation of more than 100 pigment peaks, and 79 pigment species, 41 chlorophylls, and 38 carotenoids were detected. The presence of degraded chlorophylls was a common feature, and the number and the variety of pigments differed with tea species. Generally, the numbers of chlorophyll species tended to increase with processing steps, while carotenoid species were decreased, especially by heating. Particularly in green teas, a change of carotenoid structure, conversion of violaxanthin to auroxanthin, occurred. In hot water extracts of teas, both chlorophylls and carotenoids were also detected, but the concentration of chlorophylls was less than 2% as compared with acetone extracts. The pigment compositions were compared between tea species, and they are discussed in terms of the differences in their manufacturing processes.

KEYWORDS: Chlorophylls; carotenoids; high-performance liquid chromatography; pigment analysis; teas

INTRODUCTION

Tea is a very popular drink, and about 2.5 million tons of tea are produced in the world every year (1). Various kinds of green tea have traditionally been consumed as a daily beverage in Japan. These days, eating green tea leaves is becoming popular due to the perceived health benefits (1).

Some epidemiological studies not only on polyphenols such as catechins but also on chlorophylls have been carried out. For instance, pheophytins *a* and *b* (Figure 1) of the hot water extract from green tea have potent suppressive activities against chemically induced tumorigenesis in mouse skin through suppression at the tumor promotion phase (2). On the other hand, excessive intake of pheophorbide *a*, derived from chlorophyll *a*, has been implicated in human dermatitis (1). The structures of chlorophylls and carotenoids are shown in Figures 1 and 2, respectively.

Chlorophyllase, which catalyzes the hydrolysis of phytol from chlorophyll producing chlorophyllide, plays a key role in the involvement of the first step of chlorophyll degradation. This enzyme was purified and characterized from leaf sprouts of tea (3). In certain teas, harvested leaves are withered before heat treatment, and this probably causes formation of an unstable chlorophyllide and its degradation into pheophorbide. A recent

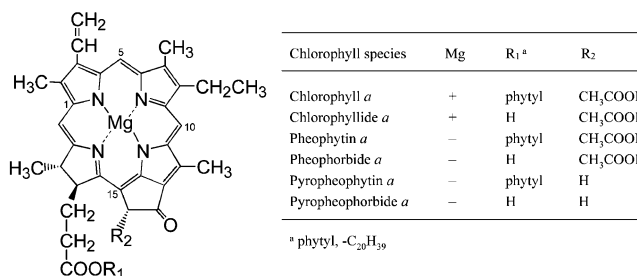


Figure 1. Structural formulas of chlorophyll species. In the case of chlorophylls *b*, the methyl group at the C₇ position is substituted by a formyl group.

paper indicates that activity of chlorophyllase remains even after being heated during the processing of tea (4). The residual activity might be different in each tea because of the differences in the concentration of the enzyme in fresh leaves and the method of processing the tea.

To our knowledge, however, there are a few papers that completely and systematically identify and compare the non-polyphenolic pigment species, e.g., chlorophylls and carotenoids, that are contained in teas or tea hot water extracts. In the present study, we analyzed the pigments extracted from several major teas produced by different manufacturing methods and fresh leaves of tea by high-performance liquid chromatography (HPLC) with a photodiode array detector and identified chlo-

* To whom correspondence should be addressed. Tel: +81-54-238-4770. Fax: +81-54-238-0986. E-mail: sbysioi@ipc.shizuoka.ac.jp.

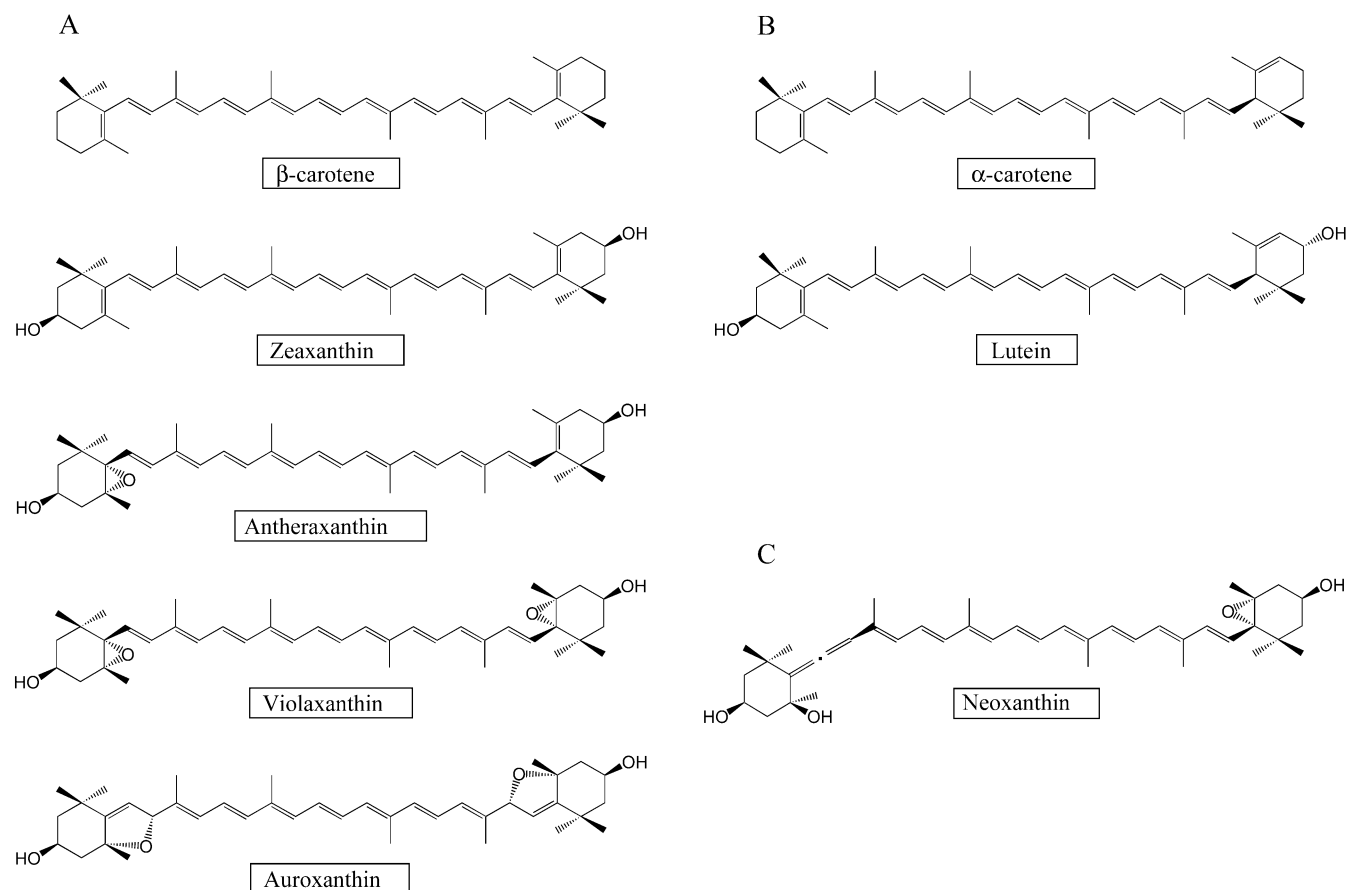


Figure 2. Structural formulas of carotenoids. (A) β,β series; (B) β,ϵ series; and (C) neoxanthin.

rophylls and carotenoids. The pigment compositions were compared between tea species and are discussed in light of the differences in their manufacturing processes. A preliminary paper of HPLC analysis of teas has been published elsewhere (5).

MATERIALS AND METHODS

Materials. Fresh leaves of tea (*Camellia sinensis* var. *sinensis* cv. Yabukita) to produce Sencha were collected in July in Shizuoka, Japan, and stored at $-30\text{ }^{\circ}\text{C}$ until extracted. Unfermented teas, Gyokuro (Okabe, Shizuoka, Japan), Matcha (Aichi, Japan), Sencha (Honyama, Shizuoka, Japan), and Hojicha (Japan); a fully fermented tea, Ceylon (Sri Lanka); a partially fermented tea, Tikuan-tin (China); and a double fermented tea, Pu-erh (China), were purchased from a local market.

Pigment Extraction. Pigments were extracted from teas or fresh leaves of tea (0.1 g) by grinding with 50 volumes of cold ($-20\text{ }^{\circ}\text{C}$) 80% aqueous acetone. In the case of extraction with hot water, tea leaves were simply soaked in 20 volumes of hot water at $90\text{ }^{\circ}\text{C}$ for 5 min. Acetone extracts (10 μL) and water extracts (40 μL) were used for HPLC analyses after filtration with a 0.45 μm syringe filter (Millipore, MA).

HPLC Analysis. HPLC was carried out with a model LC-10AT (Shimadzu, Kyoto, Japan) equipped with a column temperature controller, using a 150 mm \times 4.6 mm i.d. Waters Symmetry C₈ column (Waters, MA). Analysis of chlorophylls and carotenoids was performed according to the method of Zapata et al. (6). Pigments were eluted at a flow rate of 1.0 mL per min at $25\text{ }^{\circ}\text{C}$ with a programmed binary gradient elution system according to the method. Solvents used were A, methanol:acetonitrile:0.25 M aqueous pyridine solution (50:25:25, v/v/v), and B, methanol:acetonitrile:acetone (20:60:20, v/v/v). Separated pigments were detected spectrophotometrically with a SPD-M10A photodiode array detector (Shimadzu), measuring from 340 to 740 nm and monitoring four channels at representative wavelengths of 410, 430, 440, and 450 nm. The wavelengths used to indicate the pigments

were 410 nm for pheophorbide *a* and pyropheophorbide *a*, 430 nm for chlorophyll *a*, and 450 nm for chlorophyll *b* and carotenoids.

Pigments and Their Quantification. Chlorophyll *a*, pheophorbide *a*, pyropheophorbide *a* (Figure 1), and β -carotene (Figure 2) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Chlorophyll *b* was purified from spinach (*Spinacia oleracea*) by sugar column chromatography according to the method of Perkins and Roberts (7). Pheophytins *a* and *b* were prepared by acidic treatment of the respective chlorophylls (7). Quantities of each pigment were calculated from standard curves. Standard curves were created for five pigments (chlorophylls *a,b*, pheophytin *a*, pheophorbide *a*, and all-*trans*- β -carotene) from the relationships between concentrations and peak areas after separation by HPLC. Identification and quantification of chlorophylls such as chlorophyllide *a*, pheophorbide *a*, and pyropheophorbide *a* were carried out according to our previous method (8). Epimers of chlorophyll *a,b* and pheophytin *a* were calculated by using the respective native standards. Pheophytin *a* was used as a surrogate standard for pyropheophytin *a*.

RESULTS AND DISCUSSION

Peak Identification. Acetone and hot water extracts from fresh leaves of tea and seven representative teas were analyzed by HPLC using a photodiode array detector. Each peak was identified by comparison with HPLC retention times and absorption spectrum from the photodiode array detection data. In this study, 79 peaks, 41 chlorophylls and 38 carotenoids, were detected (Table 1; Figures 3 and 4). Of these, 34 chlorophylls and 14 carotenoids were identified from the comparison with authentic samples, peak spectroscopic data, and HPLC retention times (6, 9–11). The numbering of Table 1 applies throughout this text including the figures.

It appears that six unknown carotenoids were the *cis* type due to a peak in the 340 nm region, which indicates the presence

Table 1. Detection of Chlorophylls and Carotenoids by HPLC^a

Chlorophyll							
no.	<i>t_R</i> (min)	components			λ_{max} (nm)		
1	2.17	pheophorbide <i>b</i> sp.	(419)	438			653
2	2.31	chlorophyllide <i>b</i> sp.		465			651
3	2.41	pheophorbide <i>a</i> sp.	411		504	536	666
4	2.86	pheophorbide <i>a</i> sp.	409		507	538	666
5	3.27	chlorophyllide <i>a</i> sp.	(417)	433			666
6	3.40	pheophorbide <i>a</i> sp.	409		508	539	665
7	3.61	pheophorbide <i>a</i> sp.	411		509	540	666
8	3.64	chlorophyllide <i>a</i> sp.	(416)	432			666
9	3.81	pheophorbide <i>b</i> sp.		439			654
10	3.92	chlorophyllide <i>a</i> sp.	(416)	432			666
11	3.98		403	426			656
12	4.19	protochlorophyll <i>a</i> sp.		432			628
13	4.36		399	427			653
14	4.63	pheophorbide <i>a</i> sp.	413				667
15	5.08	pheophorbide <i>a</i> sp.	407		507		666
16	5.45			464			649
17	5.59	pheophorbide <i>a</i> sp.	411		510		666
18	6.41	pheophorbide <i>a</i> sp.	409		505	539	665
19	7.06	pheophorbide <i>a</i> sp.	411		507	539	664
20	7.33	pheophorbide <i>a</i> sp.	408				666
21	8.05	pheophorbide <i>a</i> sp.	409		507	538	665
22	12.09		(417)	429			663
23	17.97	pheophorbide <i>a</i>	409				665
24	21.59	pheophorbide <i>a</i> sp.	408				665
25	33.45			452			635
26	33.80	chlorophyll <i>b</i> sp.		461			649
27	33.90	chlorophyll <i>b</i> sp.		459			649
28	34.24	chlorophyll <i>b</i>		461		553	647
29	34.51	chlorophyll <i>b</i> epimer		461		557	649
30	34.89	chlorophyll <i>a</i> sp.		432			663
31	35.16		418				654
32	35.44	chlorophyll <i>a</i> sp.		432			660
33	35.85	chlorophyll <i>a</i>		432		584	662
34	36.19	chlorophyll <i>a</i> epimer		431			662
35	36.80		399	497			667
36	36.91	pheophytin <i>b</i> sp.		436			654
37	37.28	pheophytin <i>a</i> sp.	412				654
38	37.73	pheophytin <i>a</i> sp.	409		505	535	665
39	37.86	pheophytin <i>a</i>	408		505	535	665
40	38.24	pheophytin <i>a</i> epimer	408		505	535	666
41	39.23	pyropheophytin <i>a</i>	411		505	534	667
Carotenoid							
no.	<i>t_R</i> (min)	components			λ_{max} (nm)		
C1	11.54				420		
C2	13.35						447
C3	16.94				412	432	458
C4	18.14				417	442	470
C5	18.41				413	434	463
C6	18.58	<i>cis</i> type		407		432	449
C7	19.44			401	424	449	
C8	19.81				419	442	470
C9	20.08				414	434	462
C10	20.09	neoxanthin			421	437	465
C11	20.36			401	423	449	
C12	21.08		399		422	449	
C13	21.66	neoxanthin sp.			413	437	465
C14	21.83	violaxanthin			416	439	470
C15	21.93		398		422	451	
C16	22.51		399		422	449	
C17	22.62	neoxanthin sp.			413	437	465
C18	23.03	violaxanthin sp.			417	438	469
C19	23.37			404	422	448	
C20	26.04	antheraxanthin			(427)	446	474
C21	26.69				414	437	465
C22	26.72	auroxanthin sp.	381	402	427		
C23	27.40				414	437	463
C24	28.67	auroxanthin sp.	383	402	427		
C25	28.94			406	422	447	
C26	29.39	zeaxanthin				452	477
C27	29.63	lutein			(422)	446	474
C28	29.80			407	429	457	

Table 1 (Continued)

Carotenoid					
no.	t_R (min)	components	λ_{max} (nm)		
C29	30.04	zeaxanthin sp.		454	477
C30	30.21	lutein sp.	(424)	446	474
C31	31.06	<i>cis</i> type	(419)	446	469
C32	31.33		(419)	446	469
C33	31.47	<i>cis</i> type	(419)	442	469
C34	33.28	<i>cis</i> type	(419)	444	470
C35	33.45	<i>cis</i> type		444	472
C36	33.52	<i>cis</i> type		441	472
C37	38.51	α -carotene	(423)	447	473
C38	38.75	β -carotene		452	474

^a Blank, not identified; data in parentheses denote the shoulder.

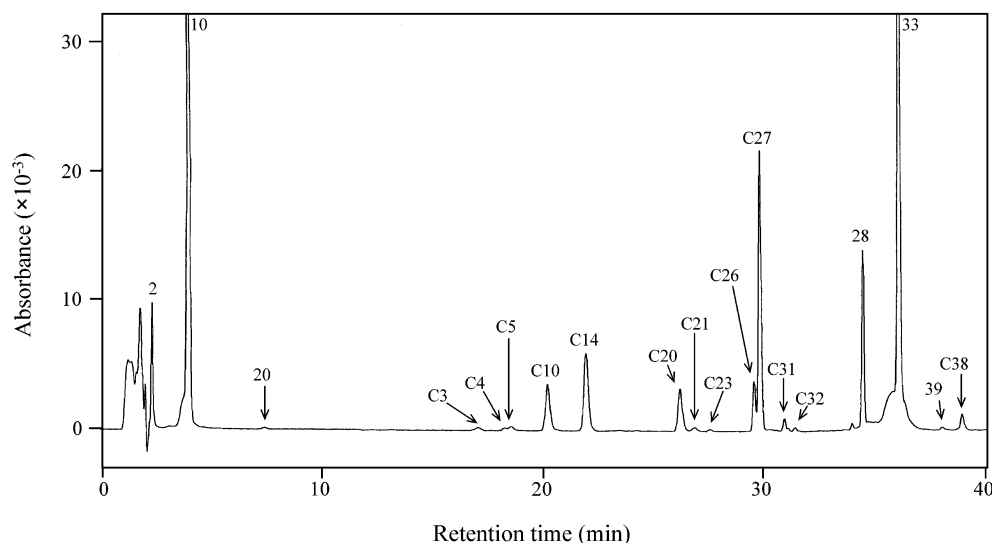


Figure 3. HPLC profile of acetone extract from fresh Sencha leaves. Detection was carried out at 430 nm. Chromatographic conditions are described in the text. Peak numbers are shown in Table 1. Nonprefixed and C-prefixed numbers denote chlorophylls and carotenoids, respectively.

of the central *cis* isomer of carotenoids (12), although a shift of the spectral bands was not tested additionally. Particularly in carotenoids, further identification of minor peaks was difficult because more than 700 species have been characterized, and individual carotenoids are present naturally in different *cis-trans* isomeric forms (13). To identify these carotenoids, further research and mass spectrometric analysis may be necessary.

Pigments of Fresh Tea Leaf. To compare the pigment compositions of tea leaves before and after processing, pigment analyses were carried out using the common and popular Sencha of Japan as a representative of teas. Fresh leaves of Sencha were provided a few days before harvest in July, 2001. An HPLC separation profile of the acetone extract from the fresh tea leaves is shown in Figure 3. From analysis of the elution data, six chlorophylls and 13 species of carotenoids were identified. Besides chlorophylls *a* and *b*, the degradation products, pheophytin *a*, chlorophyllide, and pheophorbide species, were detected but not chlorophyll epimers or pyropheophytins (Figure 3; Table 1). An accumulation of pyropheophorbide *a* has been reported in a variety of plants and algae in chlorophyll degradation during senescence (14 and references therein). We have reported that an enzyme, pheophorbidease, is involved in this reaction (15). Pheophorbidease catalyzes the conversion of pheophorbide *a* to C-13²-carboxylpyropheophorbide *a*, a precursor of pyropheophorbide *a*, and then, the precursor is decarboxylated nonenzymatically to yield pyropheophorbide *a*. The activity could not be detected in the fresh tea leaves (14). In

fact, this is consistent with the absence of pyropheophorbide in the extract from fresh tea leaves.

With respect to the carotenoids, a total of 14 species were found. Of these, five xanthophyll species, neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, and lutein, and one carotene, β -carotene, were identified (Figure 2). However, the structures of eight carotenoid species could not be determined.

Pigments of Teas. HPLC separation profiles of the acetone extracts of major teas are shown in Figure 4. The numbers of species of chlorophylls and carotenoids detected from the analyses are summarized in Table 2. Sencha, the most popular tea in Japan, is nonfermented. Sencha is made from culled, mostly young leaves by steaming and then dry kneading. The pigments, 10 chlorophylls and 12 carotenoids, were found in the acetone extract of Sencha (Table 2). The same compositions were detected in another Sencha (Kanaya, Shizuoka, Japan) with slight changes in concentrations, especially in pheophytin and pyropheophytin. The amounts of representative chlorophylls are also shown in Figure 5 for comparison. Epimers of chlorophylls *a* and *b*, which were not detected in fresh leaves, emerged. The same phenomenon was also seen in other teas. Formation of epimers of chlorophylls *a* and *b* is considered to be due to heating during processing of tea. In fact, when sweet potato leaves (*Ipomoea* spp.) were heated briefly with a microwave, the concentrations of epimers of chlorophylls *a* and *b* and pheophytin *a* increased (11). Particularly, concentrations of epimers in Sencha were highest among teas tested and comprised

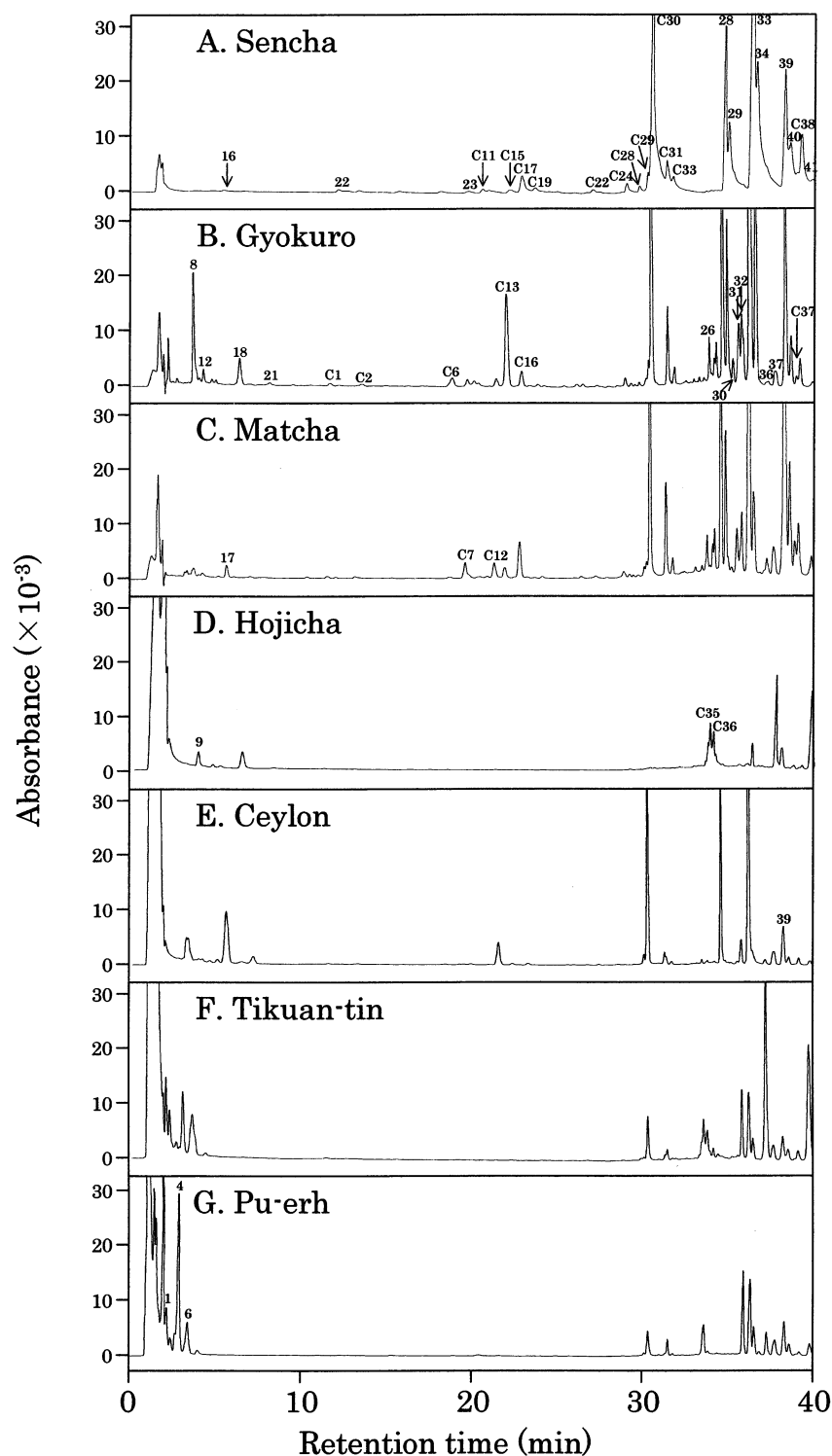


Figure 4. HPLC chromatograms of acetone extracts from teas. (A) Sencha; (B) Gyokuro; (C) Matcha; (D) Hojicha; (E) Ceylon; (F) Tikuan-tin; and (G) Pu-erh. Detection was at 430 nm. For peak numbers, see Table 1.

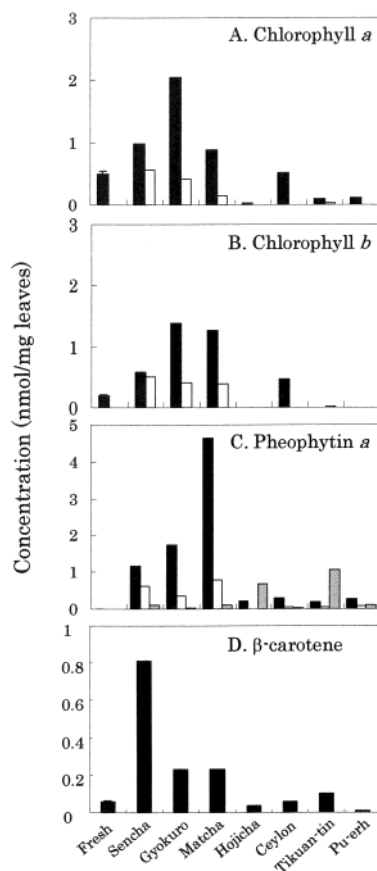
more than 35% of the total (native + epimer) (Figure 5). This is probably due to repeated heating, because tea leaves are exposed to heat at several times in the processing of Sencha. In Sencha, pheophytin *a* increased about 100-fold on a weight basis when compared to the fresh leaves, while pheophytin *b* disappeared (Figure 4A). It is clear that pheophytin *a* increased during processing to Sencha if the difference in water concentrations in the materials is taken into account. The appearance of pheophytin *a* due to heat has been well-established. The liberation of organic acids from vegetables during cooking

results in the conversion of chlorophyll *a* to pheophytin *a* (16). In addition, chlorophyll *a* is more susceptible to heat than chlorophyll *b*, and the conversion rate of chlorophyll *a* to pheophytin *a* is higher than that of chlorophyll *b* to pheophytin *b* (17–19).

A structural change of carotenoids due to heat is also observed. It was previously reported that the epoxy-containing carotenoids are more susceptible to heat than other carotenoids (11). Furthermore, 5,6-epoxides are isomerized to the 5,8-form by acids (12). There were, however, no significant stereochem-

Table 2. Chlorophyll and Carotenoid Species of Major Teas Detected after 80% (v/v) Acetone or Hot Water Extraction at 90 °C for 5 min

	chlorophylls		carotenoids	
	acetone	hot water	acetone	hot water
intact tea leaves ^a	6	0	13	0
Sencha	10	5	12	2
Gyokuro	19	6	17	1
Matcha	17	11	11	5
Hojicha	14	1	7	0
Ceylon	15	6	7	1
Tikuan-tin	13	2	8	1
Pu-erh	15	3	5	0

^a For Sencha.**Figure 5.** Comparison of the quantity of chlorophylls and carotenoid contained in acetone extracts of teas. Quantities of chlorophylls were calculated from the standard curves. (A) Chlorophyll *a*; (B) chlorophyll *b*; (C) pheophytin *a*; and (D) β -carotene. Closed column, native form; open column, epimer; shaded column, pyro type. Standard deviations were calculated from three independent experiments. Vertical bars shown are the standard deviations of more than 10%.

ical changes in neoxanthin and lutein observed in Brussels sprouts after microwave cooking (9). In the case of Sencha, violaxanthin was reduced and two auroxanthin species newly appeared, indicating that violaxanthin was converted into auroxanthin species during the processing of tea leaves. Moreover, a slight structural change may also occur in other carotenoids, neoxanthin, zeaxanthin, and lutein, because their retention times were slightly changed beyond the usual level of variations. *cis* Type carotenoids, determined spectrophotometrically, increased to about 17%, and that was 10% higher than in the fresh leaves. Our results suggest that some carotenoids were modified by heating with a more complex process

involving additional modifications rather than simple isomerization. Therefore, their structures were not determined from the results of HPLC analysis (Figure 4A), except for the conversion of violaxanthin to auroxanthin.

Gyokuro is made from fine leaves of tea bushes that are covered for a few weeks before harvest, but the processing method is the same as for Sencha. The results of HPLC analysis of the acetone extract of Gyokuro showed that 19 chlorophylls and 17 species of carotenoids were identified (Figure 4B; Table 2). The number of pigment species was much higher than that of Sencha. This is probably due to growth conditions rather than manufacturing processes because these teas are made by the same process, although they are grown under different conditions. The concentrations of chlorophylls *a* and *b* were twice as high as Sencha on a weight basis (Figures 4A,B; Figure 5). This might be derived from the shading treatment of the tea bushes. Generally, shade plants contain many more photosynthetic pigments than sun plants to harvest more light. Accompanied with an increase in chlorophyll *a*, the concentration of pheophytin *a* also increased. Each epimer of the chlorophylls and pheophytins was, however, low, and the ratio of epimer/total (native + epimer) decreased to 50% of that of Sencha. The species of carotenoids detected by HPLC analysis of Gyokuro were highest of the teas used in this investigation. In particular, carotenoid C13 (Figure 4B), which was not detected in Sencha, appeared as a remarkable peak. Unlike Sencha, α -carotene was also detectable in other Japanese teas. The decrease in the concentration of lutein and disappearance of one of the auroxanthin species occurred concurrently.

Matcha is made from Tencha, which is prepared by steaming and drying without kneading fine leaves of tea grown under covered conditions. After the veins are removed from Tencha, selected leaves are ground into fine powders to make Matcha. HPLC (Figure 4C) detected 17 chlorophylls and 11 carotenoids from the acetone extract of Matcha (Table 2). Chlorophyll *a* in Matcha was reduced by 53% as compared to Gyokuro; instead, pheophytin *a* was 270% higher (Figure 5). However, there was little difference between them in the concentration of chlorophyll *b*. Matcha was similar to Gyokuro in detectable species of carotenoids, while the quantities were different. In the extract of Matcha, peaks of C7, C16, C37 (α -carotene), and C38 (β -carotene) (Figure 5D) were higher, and the others were lower than those of Gyokuro. It remains to be determined whether these differences between Matcha and Gyokuro come from the different processing procedures of kneading or grinding to powder or something else.

Hojicha is a strongly roasted mixture of low grade Sencha and other teas. In the acetone extract of Hojicha, 14 chlorophylls and seven carotenoids were determined (Figure 4D). The number of detectable chlorophylls was higher than that of Sencha. Most nonpolar chlorophylls and carotenoids were degraded. A small amount of chlorophyll *a* and pheophytins *a,a'* appeared, but chlorophylls *a',b,b'* could not be detected. Among teas tested, Hojicha was the only tea in which the concentration of pheophytin *a'* exceeded that of pheophytin *a*. Pyropheophytin *a* was detected as usual in most of the green teas. Species and amounts of polar pigments such as pheophorbide, especially the pigments having retention times around 2–3 min, increased (Figure 4D). As compared to other green teas, the amounts of carotenoids were smaller, and lutein decreased to its measurable limit. Approximately half were the *cis* type carotenoids so far detected. These *cis* type carotenoids of Hojicha might be changed from *trans* types by heating, since new peaks appeared. This is consistent with the results of garland

chrysanthemum, in which the concentrations of *cis*-lutein and *cis*- β -carotene increased in proportion to the microwave heating time (20). It is considered that chlorophyll and carotenoid breakdown in Hojicha are greater because of the strong roast.

Ceylon is one of the black teas. In general, black teas are oxidized completely by enzyme(s) before drying. Fifteen chlorophylls and eight carotenoids were detected in the acetone extract of Ceylon tea (Figure 4E). The number of chlorophylls was higher than that of Sencha, and several pheophorbide species were found. However, epimers of chlorophylls, chlorophylls *a'* and *b'*, could not be detected. Moreover, concentrations of pheophytin and pyropheophytin were low as compared to other teas. Although the concentration of carotenoids was the same as that of Hojicha, there was only one *cis* type. The peak of C16, which was detected only in black tea and had a spectrum similar to neoxanthin, was conspicuous.

In the manufacturing of Tikuan-tin, oxidative enzyme(s) are activated by gradual evaporation of water in leaves after withering. Following pan-firing, these leaves are kneaded and then dried. We found 13 chlorophylls and eight carotenoids in the acetone extract of Tikuan-tin (Figure 4F). Concentrations of chlorophylls *a*, *a'*, *b*, *b'* were smaller than those of Sencha. The concentration of pyropheophytin *a* was the highest of the seven teas tested in this study (Figure 5). Tikuan-tin had five species of pheophytins, and in particular, pheophytin *b* species were detected. No carotenoid appeared before 29 min in the HPLC, and lutein was decreased to about 10% of Sencha. Half of the carotenoids were *cis* type like Hojicha. If the breakdown of chlorophylls and carotenoids was due only to oxidation, as in the case of black teas, pigments would remain more intact in Tikuan-tin tea than in Ceylon tea. Contrary to this assumption, Tikuan-tin had much more of the degraded chlorophylls, especially pyropheophytin, indicating that this might be caused by pan-firing rather than oxidation.

Pu-erh is one of the double fermented teas. Fermentation with fungi is added to the manufacturing process of Pu-erh. Fifteen chlorophylls and five carotenoids were separated from the acetone extract of Pu-erh (Figure 4G). The decrease in quantity of chlorophylls was similar to Hojicha, except for pyropheophytin (Figure 5). The number of carotenoids detected was the lowest among the teas tested (Table 2). In addition, the quantity of carotenoids was less than Tikuan-tin, and the amount of the *cis* type was 40%. This decrease is probably due not only to heat but also to fungal fermentation. In conjunction with the results of our HPLC analyses, chlorophyll degradation during the tea manufacturing process might occur in the same way as in other cooking processes (16–18); that is, chlorophyll converts to pheophytin, and then pheophytin degrades into pyropheophytin by heating.

Pigments of Hot Water Extract. It is known that chlorophylls are immiscible in water. There is, however, a paper that low level chlorophylls *a* and *b* are present in the blanching water of spinach (18). In fact, the hot water extract of green tea shows a slight yellow green color. Subsequently, we analyzed the pigments of teas extracted with hot water at 90 °C (Figure 6; Table 2). In green teas, Sencha, Gyokuro, and Matcha, chlorophylls and carotenoids were detectable. Of these, the largest number of species and quantity of pigments were also detected in Matcha extracts, even in hot water. The concentration of chlorophyll *a* in hot water was only about 2% of the acetone extract. In hot water extracts of Hojicha, Ceylon, Tikuan-tin, and Pu-erh, however, the pigments were scarcely detectable. It appears that the quantities of pigment extracted in hot water are related to the concentrations of the pigments in teas. Despite

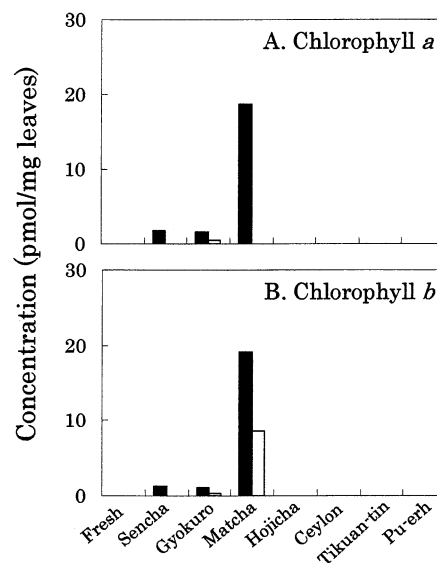


Figure 6. Comparison of the quantity of chlorophylls contained in hot water extracts of teas. Quantities of chlorophylls *a* and *b* were calculated from the standard curves. (A) Chlorophyll *a* and (B) chlorophyll *b*. Closed column, native form; open column, epimer. Standard deviations were calculated from three independent experiments. Vertical bars shown are the standard deviations of more than 10%.

the roughly equivalent quantity of chlorophyll *a* in acetone extracts of Sencha and Matcha (Figure 5), the hot water extract of Matcha had 10 times the quantity of Sencha. It is considered that chlorophylls can become miscible and exist in hot water by the action of saponins. Saponins are weak surfactants that are considered to be responsible for the bitter taste of teas and produce the froth of Matcha. In the extract of Matcha, more pigments were detected than in any other tea, because the leaves are milled into fine powders so that not only chlorophylls but probably also saponin is extracted well in hot water and thereby makes chlorophylls miscible.

The HPLC method used here was originally developed to separate the pigments from marine phytoplanktons (6). As shown in this study, this method is effective for the pigments from higher plants, in particular for the analysis of derivatives that have very similar structures such as degrading chlorophylls. In this context, we have systematically analyzed seven teas and the fresh leaf of tea and separated more than 100 peaks of pigments, and 41 chlorophylls and 38 carotenoids were roughly identified from the comparison with authentic samples and spectroscopic data. Our results showed that the number of chlorophyll species tends to increase, but carotenoid species decrease during tea manufacturing processes, especially heating. In addition, it is logical to classify major teas into two groups, green tea and others, on the basis of the concentrations of pheophytin in acetone extracts and from the presence of chlorophyll species in hot water extracts.

LITERATURE CITED

- Horie, H.; Kohata, K. Analysis of tea components by high-performance liquid chromatography and high-performance capillary electrophoresis. *J. Chromatogr. A* **2000**, *881*, 425–438.
- Higashi-Okai, K.; Otani, S.; Okai, Y. Potent suppressive activity of pheophytin *a* and *b* from the nonpolyphenolic fraction of green tea (*Camellia sinensis*) against tumor promotion in mouse skin. *Cancer Lett.* **1998**, *129*, 223–228.
- Kuroki, M.; Shioi, Y.; Sasa, T. Purification and properties of soluble chlorophyllase from tea leaf sprouts. *Plant Cell Physiol.* **1981**, *22*, 717–725.

- (4) Kohata, K.; Hanada, K.; Yamauchi, Y.; Horie, H. Pheophorbide *a* content and chlorophyllase activity in green tea. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1660–1663.
- (5) Suzuki, Y.; Shioi, Y. Chlorophyll and carotenoid analysis of tea by high-performance liquid chromatography and its application for quality control. *Proceedings of the 2001 International Conference on O-CHA (Tea) Culture and Science* **2001**, Session II, 284–287.
- (6) Zapata, M.; Rodríguez, F.; Garrido, J. L. Separation of chlorophylls and carotenoids from marine phytoplankton: A new HPLC method using a reversed phase C₈ column and pyridine-containing mobile phases. *Mar. Ecol.: Prog. Ser.* **2000**, *195*, 29–45.
- (7) Perkins, H. J.; Roberts, D. W. A. Purification of chlorophylls, pheophytins and pheophorbides for specific activity determinations. *Biochim. Biophys. Acta* **1962**, *58*, 486–498.
- (8) Doi, M.; Inage, T.; Shioi, Y. Chlorophyll degradation in a *Chlamydomonas reinhardtii* mutant: An accumulation of pyropheophorbide *a* by anaerobiosis. *Plant Cell Physiol.* **2001**, *42*, 469–474.
- (9) Khachik, F.; Beecher, G. R.; Whittaker, N. F. Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography. *J. Agric. Food Chem.* **1986**, *34*, 603–616.
- (10) Taylor, S.; McDowell, I. J. Rapid classification by HPLC of plant pigments in fresh tea (*Camellia sinensis* L) leaf. *J. Sci. Food Agric.* **1991**, *57*, 287–291.
- (11) Chen, B. H.; Chen, Y. Y. Stability of chlorophylls and carotenoids in sweet potato leaves during microwave cooking. *J. Agric. Food Chem.* **1993**, *41*, 1315–1320.
- (12) Davies, B. H. Carotenoids. In *Chemistry and Biochemistry of Plant Pigments*, 2nd ed.; Goodwin, T. W., Ed.; Academic Press: New York, 1976; Vol. 2, pp 38–165.
- (13) Oliver, J.; Palou, A. Chromatographic determination of carotenoids in foods. *J. Chromatogr. A* **2000**, *881*, 543–555.
- (14) Suzuki, Y.; Doi, M.; Shioi, Y. Two enzymatic reaction pathways in the formation of pyropheophorbide *a*. *Photosynth. Res.* **2002**, *72*, 225–233.
- (15) Shioi, Y.; Watanabe, K.; Takamiya, K. Enzymatic conversion of pheophorbide *a* to the precursor of pyropheophorbide *a* in leaves of *Chenopodium album*. *Plant Cell Physiol.* **1996**, *37*, 1143–1149.
- (16) Schwartz, S. J.; Lorenzo, T. V. Chlorophylls in foods. *Crit. Rev. Food Sci. Nutr.* **1990**, *29*, 1–17.
- (17) Schwartz, S. J.; Woo, S. L.; von Elbe, J. H. High-performance liquid chromatography of chlorophylls and their derivatives in fresh and processed spinach. *J. Agric. Food Chem.* **1981**, *29*, 533–535.
- (18) Schwartz, S. J.; von Elbe, J. H. Kinetics of chlorophyll degradation to pyropheophytin in vegetables. *J. Food Sci.* **1983**, *48*, 1303–1306.
- (19) Canjura, F. L.; Schwartz, S. J. Separation of chlorophyll compounds and their polar derivatives by high-performance liquid chromatography. *J. Agric. Food Chem.* **1991**, *39*, 1102–1105.
- (20) Chen, B. H. Studies on the stability of carotenoids in garland chrysanthemum (*Ipomoea* spp.) as affected by microwave and conventional heating. *J. Food Prot.* **1992**, *55*, 296–300.

Received for review March 4, 2003. Revised manuscript received June 4, 2003. Accepted June 6, 2003. This study was supported by grants from the Ministry of Education, Science, Sport and Culture of Japan (No. 12640631 and No. 07856).

JF030158D