Varietal Differences in the Total and Enantiomeric Composition of Theanine in Tea

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Theanine is the main amino acid component in tea. It usually constitutes between 1 and 2% of the dry weight of the tea leaves. It is as prevalent in tea as all other free amino acids combined. Both enantiomers of theanine were found to have a similar sweet taste, with little or no aftertaste. It was found that black and half-green teas (except for Formosa Oolong) contained as much, or more, theanine as green teas. No correlation was found between the absolute concentration of theanine in tea and its enantiomeric composition. An inverse correlation was found between certain grades of tea (e.g., pekoe, Flowery Orange Pekoe, etc.) and the percent of D-theanine present. This could provide the basis for a reproducible, scientific method to grade and/or evaluate teas. Theanine slowly racemizes in aqueous solution. It also undergoes hydrolysis, particularly at basic pH values. By monitoring these processes, information may be gleaned on the production, storage, handling, and shipping of tea and tea products.

Keywords: *Racemization; beverage; D-amino acid; tea grades; cyclodextrin column; column switching*

INTRODUCTION

Tea is the most widely consumed beverage in the world. The three main types of tea are (1) green (nonfermented) tea, (2) half-green or Oolong (semifermented) tea, and (3) black (fermented) tea. The term "fermented" is something of a misnomer insofar as teas are concerned. The term "oxidation" is more fitting, since tea fermentation does not require microorganisms (although they may be present in some green and half-green teas). The fermentation step consists of the oxidation of two or more tea flavanols (polyphenolic compounds) belonging to the catechin group (Wickremasinghe, 1978; Finger et al., 1992; Roberts, 1952; Harler, 1963). This oxidation step is precluded in green tea, while Oolong tea is partially oxidized, and black tea is extensively oxidized (Graham, 1992). Green tea flavor has four characteristic taste elements: bitterness, astringency, sweetness, and umami (a brothy or savory taste) (Kawamura and Kare, 1987; Teranishi, 1989; Nakagawa, 1975). The brothy, sweet, umami taste is due to amino acids, especially theanine. Theanine, also known as glutamic acid γ-ethylamide or 5-*N*-ethylglutamine, exists only in the free (non-protein) form and is by far the major free amino acid in tea (Selvendran, 1970; Neumann and Montag, 1982, 1983; Konishi and Takahashi, 1969). While most amino acids are found at trace levels, theanine represents >50% of all amino acids in tea. Earlier reports indicated that between 1 and 2% (on an average) of the total dry weight of tea leaves is theanine (Cartwright et al., 1954; Wickremasinghe, 1978; Finger et al., 1992; Millin et al., 1969; Konishi and Takahashi, 1966). The structure of theanine is similar to that of glutamine and γ-glutamyl dipeptides previously found in plants (see Figure 1) (Sasaoka and Kito, 1964). Theanine was first found in tea leaves (*Camellia sinensis* and others of the *Camellia* genus) in the early 1950s (Nagashima et al., 1957). The only other reported natural source of theanine is in the mushroom *Xerocomus badius* (Casimir et al., 1960).

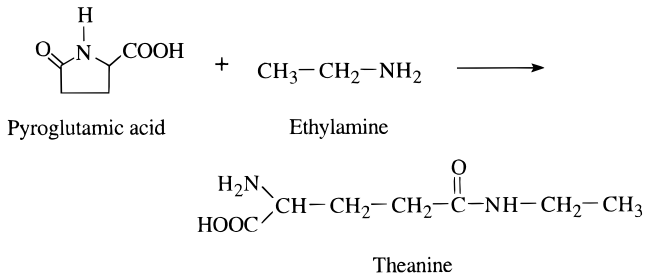

Figure 1. Reaction of pyroglutamic acid with ethylamine to form theanine.

The precursors for theanine biosynthesis are glutamic acid and ethylamine. Ethylamine is a constituent of many plant roots (Sasaoka et al., 1965; Gray, 1963; Tunmann and Linde, 1958; Serenkov, 1959) and is produced by the enzymatic decarboxylation of alanine (Crocomo and Fowden, 1970; Takeo, 1974). Biosynthesis of theanine occurs in the root of the tea plant with the aid of the enzyme theanine synthetase (also called L-glutamic acid ethylamine ligase or L-glutamate ethylamine ligase) (Feldheim et al., 1986; Sasaoka and Kito, 1964; Sasaoka et al., 1963, 1965; Wickremasinghe and Perera, 1972; Orihara and Furuya, 1990; Matsuura and Kakuda, 1990; Sasaoka, 1965; Perera and Wickremasinghe, 1971; Chakraborty et al., 1978; Konishi, 1970; Konishi et al., 1969; Konishi and Kasai, 1968a,b). The theanine is then translocated to the developing shoot tips, where it serves as the major source of soluble nitrogen for other carbon skeletal compounds (Wickremasinghe and Perera, 1972; Takeo, 1979; Kito et al., 1968; Konishi and Takahashi, 1969; Konishi, 1970; Konishi et al., 1969; Konishi and Kasai, 1968a,b). Theanine is a significant precursor for the biosynthesis of flavanols in tea leaves (Kito et al., 1968).

The flavor and fragrance components of tea can be affected by the processing and handling steps. These steps are listed and described in Table 1 (Lichtenstein, 1942; Norman, 1989). Note that each tea producer can use some, all, or a variety of these seven basic steps. All teas are graded on their physical form, age, and/or

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Table 1. Description of the Seven Processing and Handling Steps in the Production of Green, Half-Green, and Black Teas

1. Withering	Tea shoots are spread onto racks to dry until they become withered and limp and lose 50% of their moisture content. This process may take a few days in the open air, but only takes a few hours in a drum drier. All three main types of tea (green, half-green, and black) go through this process. Green tea leaves are scalded or steamed to prevent fermentation before the rolling and drying steps.
2. Rolling	Nowadays, the withered tea leaves are machine rolled for an hour or two. This breaks up the cellular structure, causing the chemical components of the leaf (oils and enzymes) to be released and mixed together. Rolling twists the leaves, which results in a delay in the rate at which the leaves release their essence in hot water during tea making. It is during this process the flavor, astringency, and color of the finished tea begin to develop.
3. Fermentation	A more accurate name for this process is oxidation. For black tea processing, the green sticky tea leaves are left for 1−4 h in a humid, cool atmosphere. This makes the tea leaves develop a coppery brown hue, together with characteristic flavor and astringency. The fermentation step progresses as far as each individual tea producer desires. The half-green tea process is a compromise between black and green tea. It is fermented briefly, once before and once after rolling. The color of the tea leaves is half-brown (half-green).
4. Drying	During this process, the tea leaves are dried in temperature-controlled chambers until they turn black and have a moisture content of ~5%.
5. Sorting and Grading	Green teas are mainly graded on the age of the leaves and their preparation. Black and Oolong teas are passed into vibrating machines, which sort the leaves through different mesh sizes. The tea grade is not necessarily an indication of quality. All black and Oolong teas are "broken" to some extent during the rolling and/or cutting steps. Since small pieces of tea leaves brew much more quickly than large ones, consistency of size is very important. There are three basic sizes of black and Oolong tea leaves, and these in turn are divided into grades (see Table 2).
6. Packaging and Shipping	Teas cannot be stored for long periods of times without loss of quality. Therefore, as soon as the tea is sorted and graded, it is packed. Sometimes, tea is shipped in paper sacks, but usually it is packed into plywood tea chests edged with metal for extra strength. These tea chests are mostly lined with paper and foil to keep the tea fresh, and packs either 30 or 45 kg tea each. To keep its aroma, loose weight tea must be stored at room temperature in an airtight container.
7. Blending	Once the tea has been bought at an auction, manufacturers blend their own special brand products for the retail market. Almost all black teas on the market are made up of a mixture of different tea types. Up to 20 or 30 different teas can be mixed into a popular blend. This is sometimes necessary to be able to guarantee the consistency (taste, smell, quality, and price) of each tea brand. A tea brand may vary in both taste and quality, even though it was produced at the same tea plantation. This variation can be attributed to the climate, handling of the tea leaves, transportation, etc. A skilled tea tester ascertains which teas are required to make up a blend consistency, and the new tea mixture is compared to blends previously produced by the manufacturer. Most tea blends contain three basic tea types: (1) Ceylon for flavor, (2) North Indian for strength, and (3) African for color and brightness. Some other tea types may be added to complete the final tea taste. In some cases, some flowers or aromatic oils have been added to the teas for look, smell, and taste.

Table 2. Grades of Green, Half-Green, and Black Teas

1. GREEN TEA[a]
- ***A. Gunpowder Tea.*** This tea consists of small tight balls of young and medium-aged leaves.
- ***B. Sencha Tea.*** This tea consists of 0.5 in. long, straight (grasslike) leaves of various ages.
- ***C. Imperial Tea.*** This is another version of gunpowder tea, consisting of older, larger, and looser leaves.
- ***D. Hyson Tea.*** This tea consists of long, twisted leaves of various ages.

2. OOLONG AND BLACK TEA
- ***A. Leaf Tea.*** This type of tea takes the longest to release its flavor. It is divided into four grades.
  - **a. Flowery Orange Pekoe** is considered the finest tea available, especially if it contains "Golden tips" or "Silver tips" (new shoot tips). The word "orange" refers to the color of the leaf tips, which are included in this grade. The word "pekoe" is Chinese for "leaf" and refers to small tea leaves which give strong brews.
  - **b. Orange Pekoe**, which has long, thin, closely twisted leaves.
  - **c. Pekoe**, which has more open leaves.
  - **d. Pekoe Souchong**, which has coarse, large leaves.
- ***B. Broken Tea.*** This type of tea is ideal for quick brewing, since it tends to release its flavor more quickly than leaf tea. The three grades available are as follows:
  - **a. Broken Orange Pekoe**
  - **b. Broken Pekoe**
  - **c. Broken Pekoe Souchong**
- ***C. Smaller Leaf Tea.*** This type of tea is excellent to use for packaged tea and tea bags, since it brews quickly. There are three grades available.
  - **a. Orange Fannings**
  - **b. Fannings** consists of small parts of the broken tea leaves.
  - **c. Dust**, which is the finest size of tea particles (made from broken leaves) used in tea bags.

[a] The table shows some examples of green teas. Most green teas are exported all over the world, while Sencha tea is used mainly in Japan.

appearance. The grading systems for green, half-green (Oolong), and black teas are given in Table 2 (Lichtenstein, 1942; Norman, 1989). During black tea manufacture, the amino acids may be oxidized by catechin *o*-quinones and undergo subsequent Strecker degradation, which leads to new aroma components (Finger et al., 1992; Saijo and Takeo, 1970; Co and Sanderson, 1970). In black tea, the importance of theanine as a flavor compound is thought to be somewhat less, since there is a breakdown of theanine into glutamic acid and ethylamine after the withering and fermentation processing steps (Feldheim et al., 1986). In other words, the highest quality black tea is thought to contain the lowest amount of theanine. All other amino acids increase significantly after the withering step, since they are breakdown products from hydrolysis of the tea

proteins (Choudhury et al., 1980; Nagashima et al., 1957). There is no single identified tea aroma and flavor constituent characteristic of green, half-green, or black tea. Instead, the aroma and flavor of tea result from a variety of volatile components, as well as amino acids. In this study, the green and half-green teas were comprised of only one tea type (i.e., tea leaves from only one plantation and one plucking), while the black teas were comprised of between one and three types of tea.

Amino acids are known to produce complex sensations in humans, although the sensations of sweet, sour, salty, bitter, and umami predominate. Of the thousands of publications on amino acids, only a fraction deal with their enantiomeric composition. Most α-amino acids contain a stereogenic center and exist as both D- and L-enantiomers. Some D-amino acids are known to have desirable and characteristic tastes. For example, some D-amino acids exhibit strong sweet tastes, while the corresponding L-enantiomers (most hydrophobic L-amino acids) elicit bitter tastes (Solms, 1969; Wieser et al., 1977; Kato et al., 1989). D-Tryptophan, D-phenylalanine, D-histidine, D-tyrosine, and D-leucine all exhibit sweet tastes that are 35, 7, 7, 6, and 4 times as strong as sucrose, respectively (Solms, 1969). Glycine and L-alanine also exhibit strong sweet tastes. The L-glutamic acid and L-aspartic acid are sour tasting in their dissociated states, while their sodium salts exhibit an umami taste. Free L-theanine and glutamic acid have been found to be the most important umami substances of green tea (Nagashima et al., 1957; Sakato, 1950; Sakato et al., 1950). The D- and L-enantiomers, as well as the racemic mixture of theanine, all taste sweet and do not seem to leave any bitter aftertastes (see Materials and Methods).

One source of D-amino acids in mammals seems to be the diet (Armstrong et al., 1993a), although the action of certain gut (intestinal) bacteria may account for their presence in some cases (Nagata and Akino, 1990; Konno et al., 1990). Racemization of amino acids may take place while the foods and beverages are being processed, stored, or prepared for human consumption (Tovar and Schwass, 1983). Food and beverage products in which D-amino acids seem to be prevalent are those that (1) have been exposed to microbial activity (aging, fermentation, etc.) (Ekborg-Ott and Armstrong, 1996; Brückner and Hausch, 1989, 1990; Gobbetti et al., 1994; Friedman, 1991; Liardon and Hurrell, 1983; Bunjapamai et al., 1982; Friedman et al., 1981; Hayashi and Kameda, 1980; Gandolfi et al., 1992; Palla et al., 1989), (2) have been highly processed (exposed to extremes of pH, heat, etc.) (Friedman, 1991; Liardon and Hurrell, 1983; Bunjapamai et al., 1982; Friedman et al., 1981; Hayashi and Kameda, 1980), and (3) contain natural sources of D-amino acids (certain seafoods) (Preston, 1987; Corrigan, 1969; D'Aniello and Giuditta, 1978; Felbeck, 1985; Matsushima et al., 1984). Several reports have been published on the presence of D-amino acids in foods and beverages (Ekborg-Ott and Armstrong, 1996; Pawlowska and Armstrong, 1994; Rundlett and Armstrong, 1994; Brückner and Hausch, 1989, 1990; Gobbetti et al., 1994; Friedman, 1991; Liardon and Hurrell, 1983; Bunjapamai et al., 1982; Friedman et al., 1981; Hayashi and Kameda, 1980; Gandolfi et al., 1992; Palla et al., 1989).

The enantiomeric purity of amino acids can be easily and routinely determined by high-performance liquid chromatography (HPLC). In this study we examined the concentrations and the enantiomeric composition of the amino acid theanine in a variety of teas. To increase the HPLC detection sensitivity for low levels of amino acids and to simplify their isolation and identification, fluorescent and/or UV-absorbing "tagging agents" are often useful (Pawlowska and Armstrong, 1994; Rundlett and Armstrong, 1994; Ekborg-Ott and Armstrong, 1996; Carpino et al., 1986, 1972; Zukowski et al., 1992, 1993; Pawlowska et al., 1993; Spöndlin and Küsters, 1994; König, 1984; Cohen and Michaud, 1993; Cohen et al., 1993; Strydom and Cohen, 1992; Schuster, 1988; Einarsson et al., 1986, 1987; Carpino, 1987; Cunico et al., 1986; Näsholm et al., 1987; Malmer and Schroeder, 1990). In this study 9-fluorenylmethoxycarbonylglycine chloride (FMOC-Gly-Cl) was used as an effective pre-column derivatizing agent for theanine. Efficient enantioselective separations can be done on this compound with a γ-cyclodextrin-bonded stationary phase LC column (see Materials and Methods) (Pawlowska and Armstrong, 1994; Rundlett and Armstrong, 1994; Ekborg-Ott and Armstrong, 1996; Armstrong et al., 1993a,b). To our knowledge, no stereoselective analyses have ever been done on theanine in tea.

MATERIALS AND METHODS

Apparatus. The HPLC system consisted of the following Shimadzu (Kyoto, Japan) devices: two pumps (LC-6A), a UV detector (SPD-6A), a fluorescence detector (RF-535), and two CR601 Chromatopac recorders. A switching valve (Rheodyne, Cotati, CA) and a 20 μL injection valve (Rheodyne) were also used. The system described above is also known as a "coupled HPLC" system or "coupled column switching" system (Armstrong et al., 1991). The mass spectral analysis was carried out with a FAB-MS system, Model 70 SEQ, Fisons Instruments (Manchester, U.K.). The ^{1}HNMR was performed with a JEOL FX-100 NMR (100 MHz) system (Peabody, MA).

Columns. The two columns used in this study were a C_{18} and a γ-cyclodextrin (γ-CD, Cyclobond II-2000), both 250 × 4.6 mm (i.d.) containing 5 μm support, supplied from Advanced Separation Technologies, Inc. (Whippany, NJ). The C_{18} column was used in the reversed-phase mode, while the γ-CD column was used in the polar−organic (also called "magic mobile phase") mode (Armstrong et al., 1993a,b; Pawlowska et al., 1993; Zukowski et al., 1993; Rundlett and Armstrong, 1994; Ekborg-Ott and Armstrong, 1996).

Standards, Derivatizing Reagent, Solvents, and Other Accessories. Standard theanine samples (pure D, pure L, and the racemic mixture) were synthesized in our laboratory from pyroglutamic acid and ethylamine according to the method introduced by Lichtenstein (1942) (see Figure 1). Five grams of pyroglutamic acid (purity >99%) was added to a 100 mL round-bottom glass flask, together with 30 mL of ethylamine. A magnetic stir bar was added, and the flask was tightly stoppered. The flask was placed on a magnetic stir plate, and the mixture was stirred for 20 days at room temperature (22 ± 2 °C). After 20 days, all excess ethylamine was removed (using a rotary evaporator). The white crystals (theanine) obtained were recrystallized from methanol (MeOH), and the round-bottom flask was stoppered and refrigerated overnight. The following day, the white theanine crystals were scraped down from the side of the flask, filtered, dried, and finally collected into a 7 mL vial with an airtight screw cap. The newly synthesized theanine was stored in a desiccator at room temperature.

To confirm the composition and purity, we performed elemental analysis, mass spectrometry (see Figure 2), and proton NMR (^{1}H NMR) of the newly synthesized theanine. The elemental analysis was performed by Galbraith Laboratories, Inc. (Knoxville, TN). Calculated from $C_7H_{14}N_2O_3$: C, 48.27; H, 8.10; N, 16.08; O, 27.55%. Found: C, 48.48; H, 8.11; N, 16.08; O, 27.33%. MS [FAB, matrix: glycerol + 0.1% ammonium chloride (NH_4Cl)], m/z (relative intensity) 175 [$(M + H)^+$, base ion]; 176 [$(M + H + 1)^+$, 10%]. ^{1}H NMR ($CDCl_3$) δ

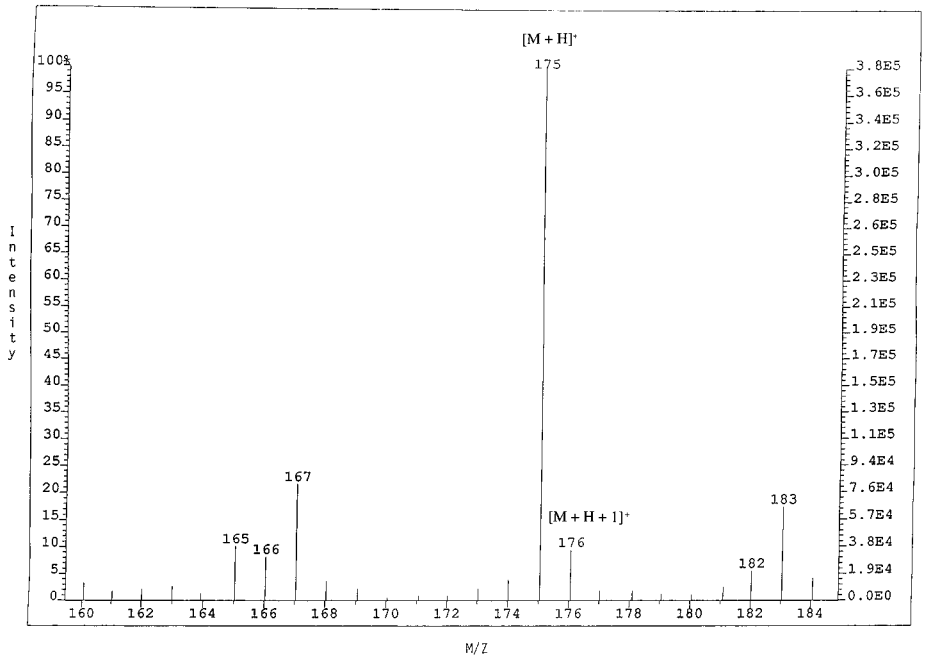

Figure 2. Mass spectrum of synthesized theanine (molecular mass 174 g/mol).

1.11 (t, 3H, $NHCH_2CH_3$), 2.15 (m, 2H, $CHCH_2CH_2COO$), 2.4 (m, 2H, $CHCH_2CH_2COO$), 3.20 (quartet, 2H, $NHCH_2CH_3$), 3.76 (t, 1H, $CHCH_2CH_2CH_2COO$). Chiral LC was used to confirm the enantiomeric purity of theanine.

We tasted the newly synthesized D,L-theanine, D-theanine, and L-theanine to determine their palatability. The racemic mixture, as well as the pure enantiomers, all exhibited similar, sweet tastes, but not as sweet as sucrose. No undesirable aftertastes were detected. This leaves the question open for further use of theanine as a sweetening or flavoring agent. However, currently there is no existing economical method for producing theanine commercially on a large-scale basis. This limits the number of studies performed on the effect of theanine on animals and humans. In one study, theanine has been shown to inhibit the convulsive action of caffeine in mice, but has proven ineffective against convulsants such as strychnine, pentetrazole, and picrotoxin (Kimura and Murata, 1971a,b; Kimura et al., 1975; Kimura and Murata, 1980).

FMOC-Gly-Cl was prepared in our laboratory according to the method introduced by Carpino et al. (1986). Solvents and organic modifiers (acetonitrile, water, acetic acid, and triethylamine) were of Omnisolve or HPLC grade and supplied by EM Science (Gibbstown, NJ) and Fisher Scientific (St. Louis, MO). Solid phase extraction cartridges (C_{18}, 0.5 g), strong cationic exchange cartridges (SCX, 600 mg), and 0.2 μm filters (all disposable) were obtained from Alltech (Deerfield, IL).

Sample Preparation, Derivatization, Chromatographic Conditions, and Analyses. All tea samples were donated by AB Kobbs Söner (Gothenburg, Sweden). Ten grams of each tea sample was analyzed in this study. Theanine is very soluble in water, but not in too many other solvents at room temperature (Lehmann and Neumann, 1974). Unfortunately, a lot of other undesirable components of the tea samples are also soluble in water. All tea samples required cleanup, consisting of various extractions, before LC injections could be done. Before derivatization with FMOC-Gly-Cl, the aqueous tea samples were adjusted to pH 4.0 with 6 M hydrochloric acid (HCl) and adsorbed onto disposable SCX cartridges. The sugars and other undesirable components (which otherwise may interfere with the derivatization procedure by reacting with FMOC-Gly-Cl) were washed out using 3 × 3-mL aliquots of water. The remaining amino acids were eluted from the SCX cartridge with a mixture of acetonitrile/sodium carbonate, 40:60 (v/v). Standards (pure D, pure L, and the racemic mixture) and pretreated tea samples were then precolumn derivatized with FMOC-Gly-Cl, as has been described in previous papers (Armstrong et al., 1993a; Pawlowska and Armstrong, 1994; Rundlett and Armstrong, 1994; Ekborg-Ott and Armstrong, 1996; Zukowski et al., 1992; Einarsson et al., 1983). The method used in this study offers a detection limit as low as the femtomole level. In aqueous buffer at pH 8.0, no detectable racemization of the amino acid occurs during the derivatization process (Zukowski et al., 1992). This reagent was prepared in the laboratory according to the directions given by Carpino et al. (1986). Derivatization was performed by first adjusting the sample to pH 8.0 using 1% (w/v) sodium carbonate (Na_2CO_3). This was followed by the addition of 30 mg of FMOC-Gly-Cl dissolved in a small amount of acetone. The reaction vial was shaken well and allowed to react at room temperature. See Figure 3 for the reaction of FMOC-Gly-Cl with theanine. After 15 min, the sample was acidified to pH 4.0 using 50% acetic acid (v/v). This stabilizes the FMOC-Gly group. After derivatization, the sample mixture was passed through a C_{18} solid phase extraction (SPE) cartridge, washed with 3 × 3-mL aliquots of water, and finally eluted with pure ethyl ether. The effluent was evaporated to dryness and redissolved in the mobile phase to be used with the reversed-phase HPLC system. In this study the C_{18}/γ-cyclodextrin (C_{18}/γ-CD) column switching method was used to analyze standard theanine and tea samples derivatized with FMOC-Gly-Cl. The eluent for the C_{18} column was acetonitrile/water/acetic acid/triethylamine, 300:700:2.0/0.5 (v/v/v/v), and the flow rate was 1.0 mL/min (see Table 3 for additional

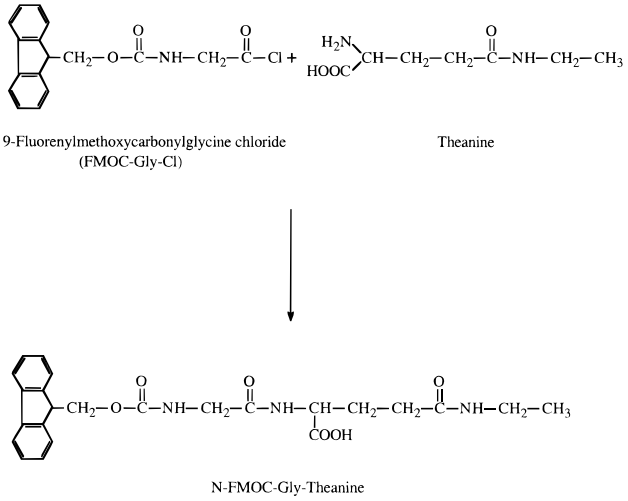

Figure 3. Reaction of FMOC-Gly-Cl with theanine.

Table 3. Chromatographic Conditions Used for the Determination of Enantiomeric Ratios of Amino Acids in Tea

	column I (RP, C_{18})		column II					
solute	mobile phase[a]	k'	column	mobile phase[b]	k'_L	k'_D	α	R_s
FMOC-Gly-theanine	300/700/2/0.5	6.73	γ-CD	1000/35/4/0.5	10.49	11.96	1.14	1.68
FMOC-Gly-2-aminoadipic acid	300/700/2/0.5	5.63						

[a] Eluent: acetonitrile/water/acetic acid/triethylamine (v/v/v/v). [b] Eluent: acetonitrile/methanol/triethylamine/acetic acid (v/v/v/v). The chromatographic conditions are given after column switching.

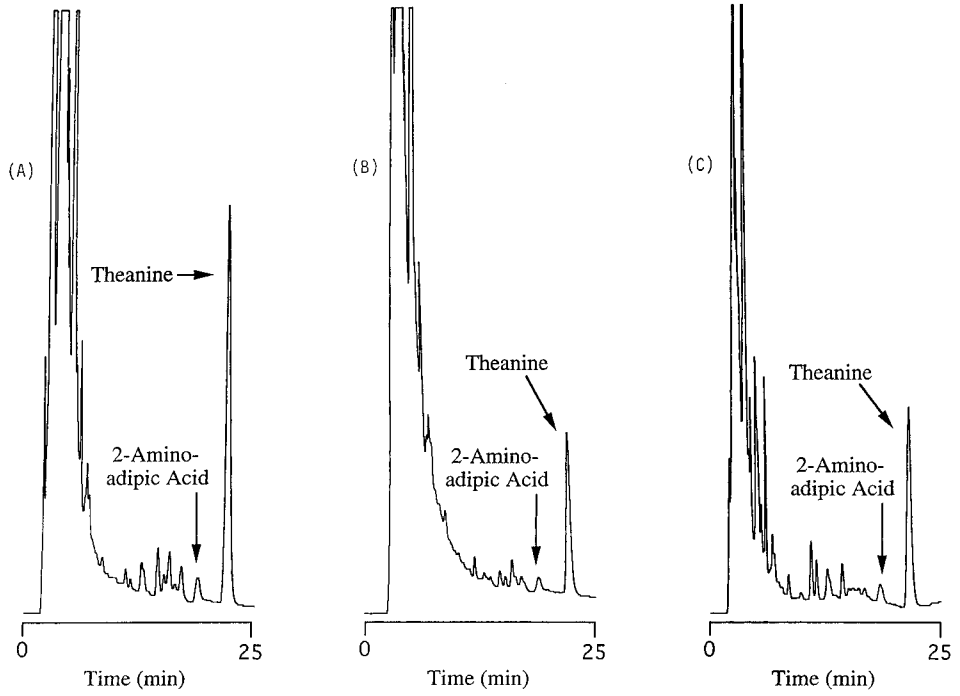

Figure 4. Representative chromatograms of theanine in different tea samples after derivatization with FMOC-Gly-Cl reagent. Column: C_{18} (used in the reversed-phase mode; see Table 3 for chromatographic conditions). Flow rate: 1 mL/min. Detection: UV at 266 nm. Samples (see Table 4): (A) Gunpowder (green) tea, (B) Jasmine FOP (half-green) tea, and (C) Keemun FOP (black) tea. The internal standard (FMOC-Gly-derivatized 2-aminoadipic acid) is also shown.

chromatographic conditions). Before 20 μL of the sample was injected onto the C_{18} column, the tea sample was filtered through a 0.2 μm disposable filter. A UV detector, set to wavelength 266 nm, was used to monitor the effluent. A total of six analyses from two different batches were performed on each tea type [each tea batch was analyzed three times (i.e., 2 $\times$ 3 = 6 determinations)]. Figure 4 shows typical achiral C_{18} reversed-phased chromatograms of FMOC-Gly-derivatized D,L-theanine in three different tea types [(A) Gunpowder, (B) Jasmine Flowery Orange Pekoe (FOP), and (C) Keemun FOP] before column switching. The internal standard used, 2-aminoadipic acid (α-aminoadipic acid), is also shown (see Table 3 for chromatographic details). The column switching valve was turned for 1−10 s at the maximum of the eluted peak of interest, and a small portion of the peak (FMOC-Gly-theanine) was introduced onto the chiral γ-CD column. This column-switching method has been described in previous papers (Armstrong et al., 1993a; Pawlowska and Armstrong, 1994; Rundlett, and Armstrong, 1994; Ekborg-Ott and Armstrong, 1996). The γ-CD column was used in the polar−organic mode. The mobile phase consisted of acetonitrile/methanol/triethylamine/acetic acid, 1000:35:4/0.5 (v/v/v/v), and the flow rate was 1.0 mL/min (see Table 3). The effluent was monitored with a fluorescence detector operated at λ_{ex} = 266 nm and λ_{em} = 315 nm. Figure 5 shows the separation of D,L-theanine in six different tea samples [(A) Sencha , (B) Gunpowder, (C) Formosa Oolong, (D) Lapsang Souchong, (E) Rosen, and (F) Keemun FOP] on the γ-CD column after column switching. When enantiomeric ratios are determined or extremely low levels of D-amino acids (<1%) are quantitated in the presence of the larger amounts of the L-enantiomers, it is often inaccurate to simply use the data from the automated peak integrator. This is because the linear dynamic range of the detector is sometimes exceeded for the larger peaks (i.e., L- vs D-peaks). In this case, the larger peaks must often be quantitatively measured a second time after accurate serial dilutions. For the tea samples containing only trace amounts of D-theanine, proper dilutions (~5−10-fold) were used to measure the correct amount of the D- and L-enantiomers, respectively.

Temperature (Racemization) Study. To see if there were any racemization of theanine taking place, six tea samples (two green, two half-green, and two black teas), as well as standard L-theanine, were heated in a water bath at 45 ± 2 °C for 120 h. All solutions had a measured pH of 5.0. The samples were precolumn derivatized with FMOC-Gly-Cl and analyzed on the coupled column system (C_{18}/γ-CD) according to the experimental scheme mentioned above. The increase in D-theanine was reported for each sample analyzed. See Figure 6, Table 3, and Results and Discussion for further information.

Hydrolysis Study. Theanine can be broken down into glutamic acid and ethylamine. To see if pH has any significant impact on the loss of absolute amount of theanine in nature, hydrolysis studies were done at different pH values. Three different tea types (Gunpowder, Jasmine FOP, and Ceylon Pekoe), as well as L- and D,L-theanine were divided into three parts each, and adjusted to different pH values (pH 3, 7, and 11) using 0.1% triethylammonium acetate buffer. Amount of glutamic acid formed with respect to loss in theanine [i.e., glutamic acid/(glutamic acid + theanine) $\times$ 100%] was reported over a period of 336 h. Both primary amino acids (glutamic acid and theanine) were precolumn derivatized with FMOC-Gly-Cl. No cleanup was necessary before the samples were analyzed on the reversed-phase C_{18} column using the scheme given above. See Figure 7 and Results and Discussion for further information.

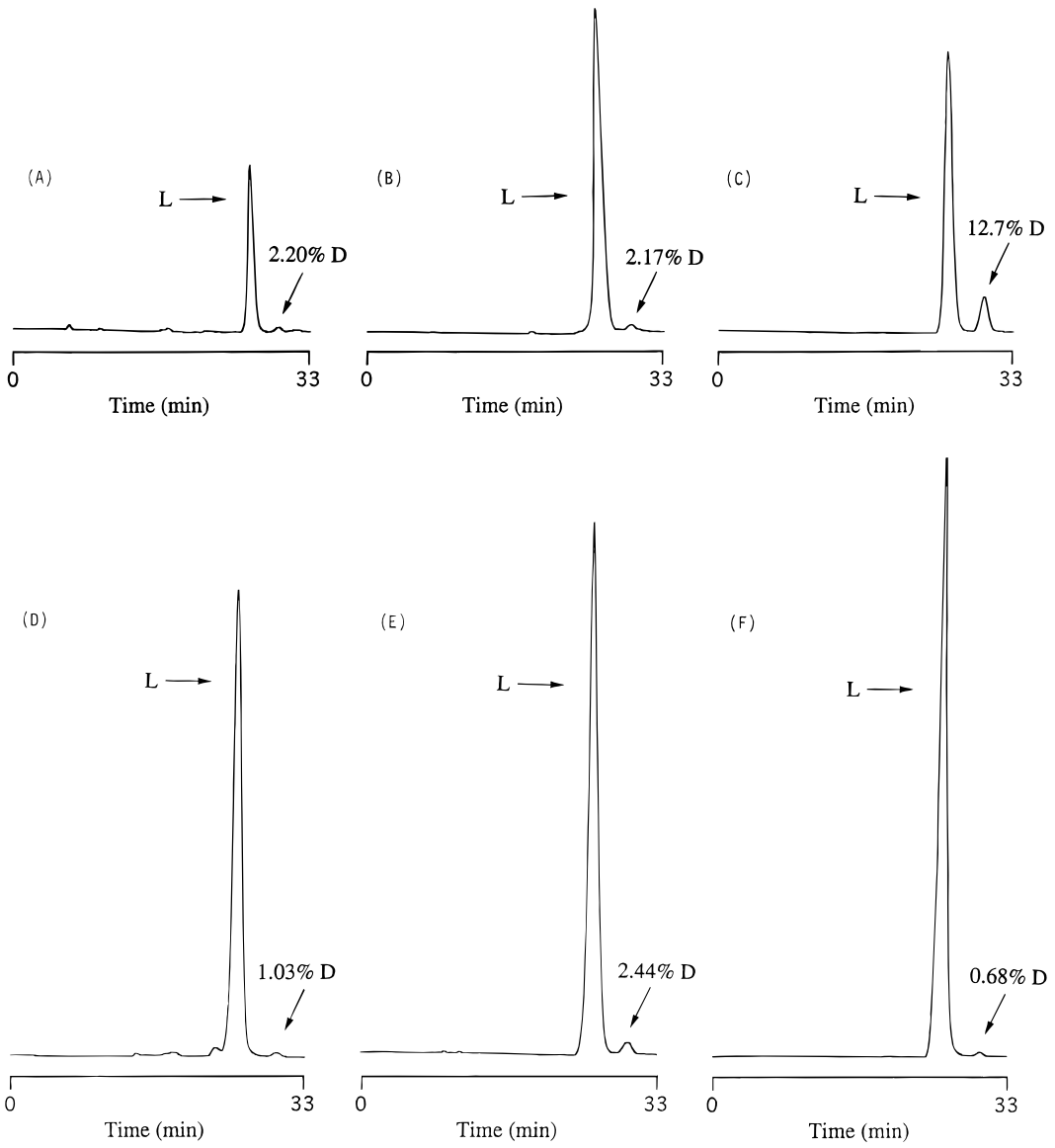

Figure 5. Determination of enantiomeric composition of theanine in tea samples using FMOC-Gly-Cl derivatizing agent and the column switching technique. Column: γ-CD (used in the polar−organic mode; see Table 3 for chromatographic conditions). Flow rate: 1 mL/min. Detection: fluorescence, λ_{ex} = 266 nm and λ_{em} = 315 nm. Samples (see Table 4): (A) Sencha (green) tea, (B) Gunpowder (green) tea, (C) Formosa Oolong (half-green) tea, (D) Lapsang Souchong (black) tea, (E) Rosen (black) tea, and (F) Keemun FOP (black) tea.

RESULTS AND DISCUSSION

Tea is a natural product. The chemical composition of tea can vary depending on season, climate, altitude, tea variety, age of the leaf, position of the leaf on the harvested shoot, horticulture practices, processing of the plant material, shipping, handling, storage, as well as many other factors (Graham, 1992; Konishi and Takahashi, 1969). Moisture and heat will cause stored tea to deteriorate. Loss of astringency and flavor may occur due to lipid hydrolysis and autoxidative reactions that cause losses of amino acids (particularly theanine), sugars, and other flavor compounds (Stagg, 1974). To prevent undesirable changes (loss of desirable flavors and tastes, or addition of undesirable flavors and tastes) tea should be stored in airtight containers.

Theanine has been known to play an important enhancing role in the quality and characteristics of green tea (Nakagawa, 1970, 1975; Nagashima et al., 1957). Table 4 summarizes the results obtained for the total amount as well as for the enantiomeric compositions of theanine in 17 different tea samples. The absolute amount and the enantiomeric composition of theanine were different for each of the tea samples examined in this study. The average amount of total theanine was 1.37 g/100 g of tea, with Formosa Oolong (0.60 g/100 g of tea) having the lowest concentration and Yunnan (2.38 g/100 g of tea) the highest (see Table 4). These values are in the general range of theanine levels reported previously. Interestingly, the black teas had roughly equivalent or higher levels of theanine compared to the green and half-green teas. Thus, it is likely that theanine imparts important flavor characteristics to black teas, as well as to green and half-green teas. This is contrary to some previously reported results (Nakagawa, 1970, 1975; Nagashima et al., 1957). It is also interesting that different grades of the same type of tea from the same location (i.e., Ceylon Broken and Ceylon Pekoe from Sri Lanka) can have significantly different amounts of theanine, as well as different enantiomeric ratios (Table 4).

The average relative level of D-theanine determined in this tea study was 1.85%, with Cherry Blend (0.21%) containing the smallest relative amount and Formosa

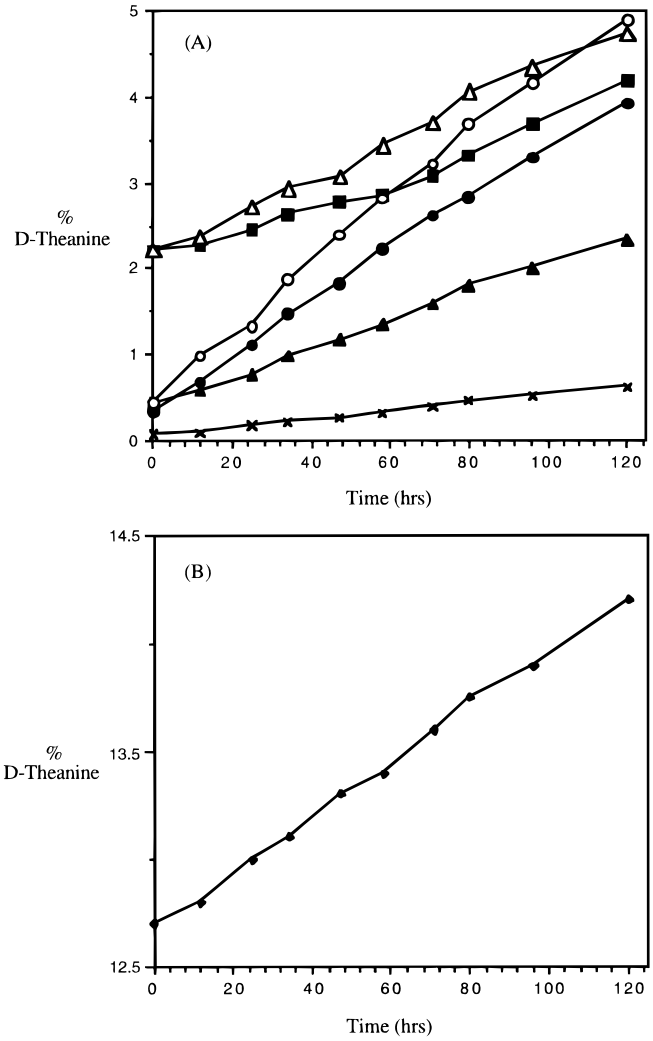

Figure 6. Change in the enantiomeric ratio of theanine during heating in a water bath at 45 ± 2 °C, using the column switching method. The chromatographic conditions used for the determinations are listed in Table 3. (A) Samples (see Table 4): (×) L-theanine standard, (■) Gunpowder (green) tea, (△) Sencha (green) tea, (▲) Jasmine (half-green) tea, (●) Ceylon Pekoe (black) tea, and (○) Darjeeling FOP (black) tea. (B) Sample: (◆) Formosa Oolong (half-green) tea.

Oolong (12.7%) the largest. Formosa Oolong is an interesting tea. In addition to having the highest amount of the D-enantiomer of theanine, it also contained the smallest absolute amount of theanine (0.60 g/100 g of tea). A possible explanation for this distribution may be found in the handling of the Formosa Oolong tea. Formosa Oolong is a half-green tea, consisting of only one type of tea (tea leaves from only one plantation). Since the Oolong tea is partially oxidized during the fermentation step, there may be some restricted growth of microorganisms, such as yeasts or bacteria in the tea leaves (Harler, 1963; Graham, 1992). These microorganisms may either utilize the L-theanine as a source of energy (leaving behind the accumulated D-enantiomer) or racemize the L-theanine while it is being used (i.e., microbial fermentation). Another possible explanation may be the degradation of L-theanine by tea enzymes. More extensive studies will have to be done to determine the reason for the high percentage of D-theanine in Formosa Oolong tea.

An interesting trend relating to the enantiomeric composition of theanine was found in Table 4. The teas that had the lowest relative amounts of D-theanine (<1%) were always of pekoe or FOP grades. The word

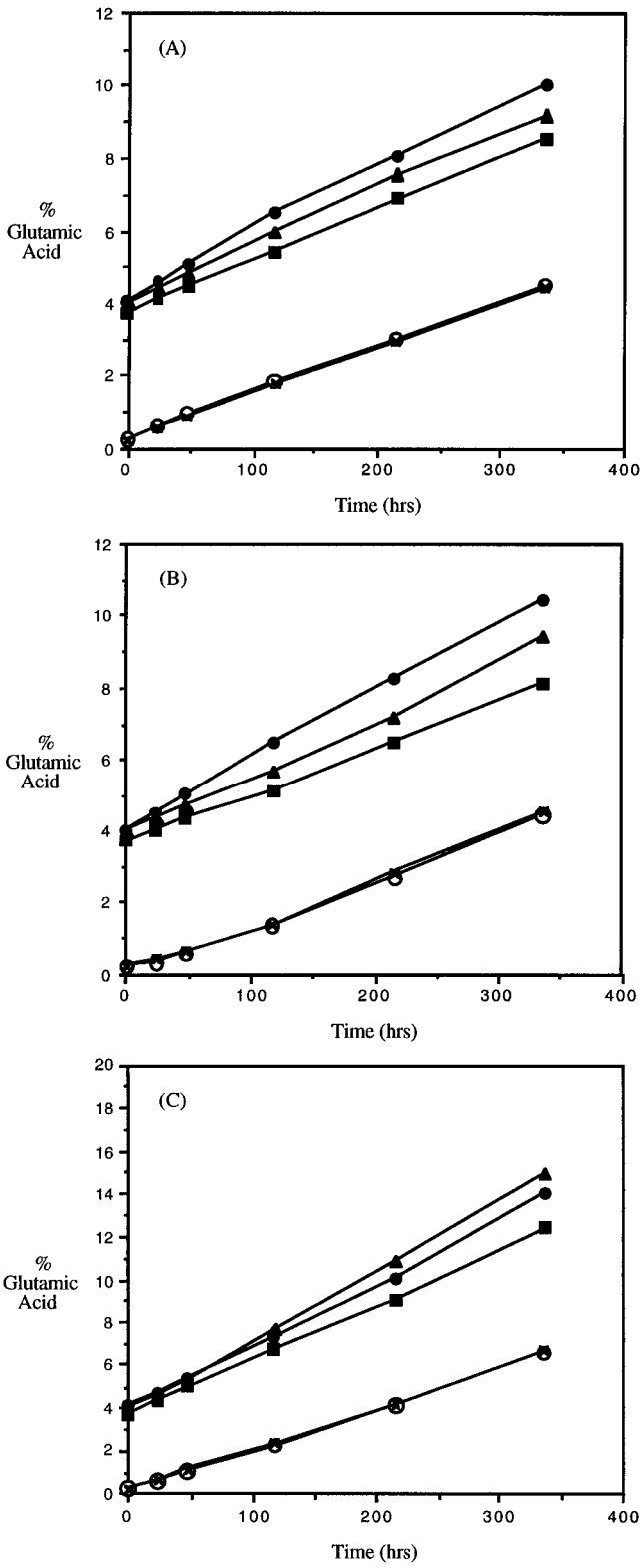

Figure 7. Representative graphs of the hydrolysis experiment performed at various pH values. The change (increase) in absolute amount of glutamic acid is shown. Samples (see Table 4): (○) D,L-theanine standard, (×) L-theanine standard, (■) Gunpowder (green) tea, (▲) Jasmine FOP (half-green) tea, and (●) Ceylon Pekoe (black) tea. Hydrolysis was at (A) pH 3, (B) pH 7, and (C) pH 11.

"pekoe" is Chinese for "leaf" and refers to small tea leaves which give strong brews. FOP is considered the finest tea available, especially if it contains new shoot tips (golden tips or silver tips). The word "orange" refers to the color of the leaf tips. It may take many years to become a proficient tea grader. The position of the

Table 4. Enantiomeric Composition and Total Amount of the Amino Acid Theanine in Various Tea Samples

tea name	tea type	country where produced	theanine %D[a]	SD[b]	total amount[c,d] (g of theanine/100 g of tea)
African Flower[e]	black	Kenya	0.54	0.05	1.30
Assam FOP[f]	black	India	0.49	0.03	1.05
Ceylon Broken	black	Sri Lanka	2.30	0.09	1.32
Ceylon Pekoe	black	Sri Lanka	0.34	0.04	2.20
Cherry Blend FOP[f]	black	India/China	0.21	0.04	2.04
Darjeeling FOP[f]	black	India	0.45	0.03	1.45
Earl Grey[e]	black	China	0.42	0.03	1.07
Formosa Oolong	half-green	Taiwan	12.7	0.4	0.60
Georgian FOP[f]	black	Georgia	0.46	0.02	1.16
Gunpowder	green	China	2.20	0.20	1.78
Jasmine FOP[f]	half-green	China	0.45	0.04	1.72
Keemun FOP[f]	black	China	0.65	0.06	1.12
Lapsang Souchong	black	China	1.04	0.12	0.82
Lemon Blend	black	India/Sri Lanka	2.70	0.23	1.26
Rosen	black	China	2.46	0.24	1.03
Sencha	green	Japan	2.20	0.09	1.05
Yunnan	black	China	1.79	0.16	2.38

[a] The number shown is the mean of six separate enantiomeric analyses performed on each tea type [i.e., D/(D + L) × 100%] using a γ-CD column. [b] The average deviation form the mean for six separate analyses of D-theanine. Three determinations from two different tea batches were performed (i.e., 3 × 2 = 6 determinations). [c] This number represents the total amino acid content regardless of chirality (i.e., L plus D enantiomers) and is the average of six determinations performed on a C_{18} column. (Three determinations from two different tea batches were performed.) [d] 2-Aminoadipic acid (α-aminoadipic acid) was used as the internal standard for all quantitative measurements. [e] The low relative level of D-theanine seemed to indicate that African Flower and Earl Grey are either pekoe or FOP grade teas. Indeed, further investigation revealed that they are FOP teas. [f] FOP, Flowery Orange Pekoe.

Table 5. Variations in Two Different Lots of the Same Tea Reflect Differences in Total and Enantiomeric Amounts of Theanine

	theanine			
tea type	range of total amount[a–c] (g/100 g of tea)	SD[d]	range of D-theanine[e] (%)	SD[c,d]
Sencha	0.93−1.18	0.09	2.09−2.32	0.09
Formosa Oolong	0.43−0.73	0.11	12.1−13.3	0.4
Yunnan	2.29−2.46	0.06	1.58−1.98	0.16

[a] These numbers represent the range for total amount of theanine regardless of chirality (i.e., L plus D enantiomers) for six samples analyzed on a C_{18} column. (Three determinations from two different tea batches were performed.) [b] 2-Aminoadipic acid was used as the internal standard for the quantitative measurements. [c] The chromatographic conditions are the same as those listed in Table 3. [d] This number is the average deviation from the mean for six separate determinations. Each tea batch was analyzed three times (i.e., 2 × 3 = 6 determinations). [e] These numbers represent the range for the enantiomeric composition of theanine (i.e. D/(D + L) × 100%) for six analyses performed on a γ-CD column. Three determinations from two different tea batches were performed.

leaves on the tea plant, the size of the tea leaves, and their physical appearance must be taken into account when teas are graded. It appears from the results (Table 4) that there may be a quantitative relationship between the grade of tea and the relative level of D-theanine. Generally, the pekoe and FOP grades of tea contained the lowest percent of D-theanine.

We also examined the natural variation of theanine within a single type of tea coming from the same producer (i.e., lot-to-lot or batch-to-batch variations). Table 5 summarizes the results for total amount and enantiomeric composition of theanine in three different tea types: Sencha (green) tea, Formosa Oolong (half-green) tea, and Yunnan (black) tea. Sencha is representative of a green tea. Formosa Oolong tea was chosen because it tended to have the lowest absolute amount of theanine and the highest relative amount of D-theanine. The Yunnan tea was chosen since it had the highest absolute amount of theanine of all samples analyzed. Different batch samples of each tea were analyzed three times each. The results show that batch-to-batch variations occur within the same type of tea. Formosa Oolong tea showed the most variability in the absolute amount of theanine (0.30 g/100 g of tea). Sencha tea and Yunnan tea had differences of 0.25 and 0.17 g/100 g of tea, respectively, in the absolute amounts of theanine. The enantiomeric differences were 1.2% for Formosa Oolong tea, 0.40% for Yunnan tea, and 0.23% for Sencha tea. Consequently, one should expect at least this much variation in the theanine concentrations (both absolute and enantiomeric) in different types of tea. As can be seen in Table 4, the variation in different types and grades of teas generally exceeds that found between batches of the same type (and grade) of tea from the same location. There can be as much as a 4-fold difference in the absolute amounts of theanine in different tea types and as much as a 60-fold difference in relative amounts of D-theanine.

The significant amounts of the D-enantiomer found in a number of commercially available teas suggest that enantiomeric ratios might be useful as an indicator for long-term storage, or possibly for shipping, handling, and processing procedures of each type of tea. This could be particularly true for tea-based drinks that are sold already brewed and bottled. To support this hypothesis (to see if solution racemization of theanine takes place), six tea samples (two green, two half-green, and two black teas), as well as standard L-theanine, were heated in a water bath at 45 ± 2 °C for a measured period of time. The results reported in Figure 6 show significant increase in the level of D-theanine in all samples analyzed. Linear relationships were found (D-theanine vs time) for all samples studied, although the racemization of theanine was much lower in the standard L-theanine sample compared to any of the "real-world" teas. This suggests that other tea factors are involved in the racemization of theanine in teas.

In nature, theanine is also broken down into glutamic acid and ethylamine. Consequently, the hydrolysis of theanine was examined at different pH values. Three different tea types (Gunpowder, Jasmine FOP, and Ceylon Pekoe), as well as L- and D,L-theanine were divided into three parts each and adjusted to different pH values (pH 3, 7, and 11). Analyses were done

regularly over a period of 336 h. This study measured the amount of glutamic acid produced with respect to loss of theanine [i.e., glutamic acid/(glutamic acid + theanine) × 100%] vs time. Figure 7 summarizes the results of the hydrolysis study. There seems to be a linear relationship for glutamic acid formation with time at different pH values. There was little difference in hydrolysis of theanine between pH 3 and 7. However, the samples at pH 11 showed a far greater amount of hydrolysis. The standard theanine samples, dissolved in buffered aqueous solutions, did not show as much hydrolysis as the individual tea samples. Once again, this indicates that there may be other components in tea that accentuate reactions involving theanine (in this case, hydrolysis).

CONCLUSIONS

Chiral discrimination plays an important role in odor, aroma, and taste perception, as well as in biological structure−function relationships. Free D-theanine was found at significant levels in all tea samples analyzed in this study. Contrary to previous reports, theanine was as prevalent in black and Oolong teas as in green teas. Therefore, theanine, which is known as an important taste element in green tea, must also play an important role in the taste characteristics of Oolong and black teas. Each of the pure enantiomers and the racemic mixture of theanine have a similar, sweet taste, with no bitter aftertaste.

There does not seem to be any correlation between the absolute amount of theanine in tea and its enantiomeric composition, with the possible exception of Formosa Oolong. On the other hand, there appears to be a correlation between enantiomeric composition of theanine and certain grades of tea. Lower percentages (<1%) of the D-enantiomer of theanine were always associated with pekoe or FOP teas. This suggests that enantiomeric analysis may be a useful tool in the grading of teas.

The hydrolysis of theanine is accelerated in basic solutions. Theanine also racemizes in aqueous solution. The theanine in authentic tea samples both racemized and hydrolyzed more quickly than did standards of theanine dissolved in aqueous or buffered solutions. This could be useful in evaluating storage, handling, and shipping practices for tea and tea-based products.

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