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Analysis of Theaflavins and Thearubigins from Black Tea Extract by MALDI-TOF Mass Spectrometry

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Black tea contains two major groups of pigments, theaflavins (TFs) and thearubigins (TRs). TFs contain a bis-flavan substituted 1,2-dihydroxy-3,4-benzotropolone moiety. Unlike the TFs, TRs have not yet been characterized. The chemical structure of the TRs remains a mystery. The present paper reports our effort to study the structure of TFs and TRs using delayed pulsed ion extraction of ions generated via the matrix-assisted laser desorption ionization (MALDI) technique, on line with a Linear time-of-flight (TOF) mass spectrometer. Spectra of standard TFs show not only pseudomolecular ions but also ions resulting from fragmentation. The analysis of MALDI-TOF spectra of black tea fractions shows the structure of some TRs, which are similar to those of TFs because the same loss of mass is observed.

KEYWORDS: Black tea; theaflavins; thearubigins; MALDI-TOF; delayed extraction; fragmentation

INTRODUCTION

Considerable interest developed in the past few years as to the potential health-promotion properties of tea (*Camellia sinensis*, Theaceae). Numerous epidemiological studies link the drinking of tea to a reduction of the risk of cardiovascular disease and cancer in humans, but the results are not consistent (*1*−*5*). The health beneficial effects of tea have been attributed to the antioxidant and other properties of some of the polyphenolic components, particularly the catechin (flavan-3-ol) derivatives (*1*−*5*). These catechin derivatives may help protect against heart disease and cancer by minimizing the impact of free radical damage to cells and tissues, modulating endothelial functions (*6*), inhibiting protein kinase activities that are key to cell growth or inflammation (*5*), and inhibiting other enzymes, such as nitric oxide synthase (*7*), cyclooxygenase, and lipoxygenase (*8*). Black tea extracts containing thearubigins (TRs) were recently reported to effectively protect against the paralytic actions of botulinum neurotoxins (*9*).

Three types of tea are produced from the leaves of *Camellia sinensis*, green tea (nonfermented), oolong tea (semi-fermented), and black tea (fermented). The fermentation of tea leaves induces enzymatic oxidation of flavan-3-ols and leads to the formation of two major pigments in black tea, theaflavins (TFs), and TRs. Identification and characterization of these compounds are necessary to increase the understanding of their biological activity. TFs, comprising about 3−5% (wt/wt) of extract solids (*10*), are well characterized and, in solution, exhibit a bright orange-red color. Their structures contain a benzotropolone nucleus formed by co-oxidation of selected pairs of catechins, one with a *vic*-trihydroxyphenyl moiety, and the other with an *ortho*-dihydroxyphenyl structure (*11*). TRs, red-brown or dark-brown, which comprise about 20% (wt/wt) of extracted solids (*10*), are heterogeneous polymers, the structures of which remain poorly characterized. Several classifications of TRs have been described. The first one classifies TRs into three groups according to their solubility in different solvents. SI TRs are extractable into ethyl acetate, whereas SIa and SII TRs, which are those that remain in the aqueous phase, are more soluble in diethyl ether (*12*). Another classification method uses the chromatographic behavior of different TRs in reverse-phase high performance liquid chromatography (HPLC) and confirms the difficulty involved in their isolation and analysis (*13*). Group I encompasses TRs excluded from HPLC column, group II is resolved TRs, and group III is TRs remaining unresolved and eluted as a "hump." A previous study (*14*) using chromatography and chemical degradation of isolated fractions indicates that they are heterogeneous polymers of flavan-3-ols and flavan-3-ols gallate with C4−C8 or C6 and C6′−C6′ (B-ring) interflavonoid linkages. Many studies use an in vitro enzymatic model fermentation system either combined with or without reverse-phase HPLC to study formation of chromatographically resolvable and unresolvable thearubigin-like substances (*15*−*21*). They show that tea polyphenol oxidase, peroxidase, and pH play key

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roles in the formation of TFs and TRs from catechins. Polyphenol oxidase produces TFs and chromatographically resolved TRs and peroxidase produces unresolved TRs (*18*). It has been demonstrated that TRs are formed by the oxidation and reaction of two gallocatechins (epigallocatechin (EGC) and epigallocatechin gallate (EGCG)) (*16*). The most recent study suggests that tea peroxidase takes part in the formation of TRs through the oxidation of TFs in the presence of H_2O_2 (*21*, *22*). Therefore, characterization of TR-like molecules is tedious and information about their structure is difficult to obtain and always incomplete.

Matrix assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry has been used for food analysis and has many advantages, such as speed and ease of use, and it is highly sensitive (*23*). Direct analysis of a food extract is possible because MALDI-TOF is tolerant of impurities and allows the simultaneous determination of masses in complex mixtures of low and high molecular weight compounds. MALDI-TOF was originally developed for large biomolecules (*24*). It is generally known as a "soft" ionization that produces almost exclusively intact pseudomolecular ion species without ion fragmentation at the time of the desorption event (pseudomolecular ion formation). However, studies (*25*) show that a significant degree of metastable ion fragmentation occurs. Their detection depends on the configuration of TOF instrumentation. If a time delay is incorporated between ion formation (desorption event) and ion extraction, ions in the source are allowed to fragment into smaller ions and neutral species before their extraction and acceleration in the tube. These types of fragmentation have been described for peptides and proteins with cleavage of amide function (*26*).

Catechins in tea have already been analyzed by MALDI-TOF (*27*), but no study mentions the MALDI-TOF analysis of TFs and TRs. This paper reports the ability of MALDI source on line with a linear TOF using delay pulsed ion extraction (DE) to give structural information about compounds contained in black tea and allows us to propose polymerized and oxidized structures.

MATERIALS AND METHODS

General Experimental Procedures. Epicatechin, ProteoMass peptide MALDI-MS calibration kit and the matrixes 2,4,6-trihydroxyacetophenone (THAP), and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma (St. Louis, MO). Theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, theaflavin-3,3′-digallate, theaflavate A, theaflavate B, and theadibenzotropolone A were synthesized and previously reported by our group (*22*, *28*). Methanol, water, and acetone are HPLC grade, purchased from Fischer Scientific (Suwanee, GA).

Instrumentation. MALDI-TOF mass spectra are acquired on a linear TOF instrument using DE (Ciphergen Biosystems, Inc., Fremont, CA). Samples are deposited on a gold chip array (eight spots) (Ciphergen Biosystems, Inc., Fremont, CA). Compounds cocrystallized with matrix are desorbed and ionized by a nitrogen laser (wavelength 337 nm; 4 ns pulse width) and extracted by 3 kV pulse voltage with time-delayed extraction (between 350 and 380 ns) before entering the time-of-flight mass spectrometer and accelerated under 20 kV. All spectra are recorded with a detector voltage of 2.9 kV and are the average result of 65 laser shots. Laser intensity and sensitivity of detector are variable. MALDI-TOF is calibrated with an 8 point external calibration using [epicatechin + H^+] (*m*/*z* 291), [theaflavin + H^+] (*m*/*z* 565), [bradykinin fragment 1−7 + H^+] (*m*/*z* 757.4), [theaflavin-3,3′-digallate + H^+] (*m*/*z* 869), [angiotensin II (human) + H^+] (*m*/*z* 1046.5), [$P_{14}R$ (synthetic peptide) + H^+] (*m*/*z* 1533.9), [ACTH fragment 18−39 (human) + H^+] (*m*/*z* 2465.2), and [insulin oxidized B chain (bovine) + H^+] (*m*/*z* 3494.6). In interpretation of spectra, peaks at *m*/*z* ratio lower than 500, too close to the reagent interferences, are not taken into account.

MALDI-TOF MS. The purchased matrixes, THAP (*29*) and α-cyano-4-hydroxycinnamic acid (CHCA), are used without further purification. They are dissolved in a mixture of methanol and water (1:1; v/v) to obtain variable concentrations (saturated for CHCA and 10 or 5 mg/mL for THAP). TF standards or black tea fraction (about 1 mg) are dissolved in 1 mL of methanol and mixed to the matrix solution (1:10; v/v). A 1-μL aliquot of this mixture is put on a spot of the gold array and dried at room temperature.

Preparation of Black Tea Fractions. Yunnan black tea (908 g), purchased from the local Chinese supermarket, was extracted with 80% acetone (5 times) at room temperature for two weeks. The extract was concentrated to dryness under reduced pressure, and the residue was dissolved in water and partitioned with chloroform, ethyl acetate, and *n*-butanol. The ethyl acetate fraction was subjected to Sephadex LH-20 eluted by an acetone/water solvent system (30%-60%) to give 14 fractions.

RESULTS AND DISCUSSION

Many compounds contained in black tea have been precisely identified. The first well-known group are the TFs. TFs encompass molecules containing a benzotropolone nucleus formed by an oxidative reaction between the *vic*-trihydroxybenzene and *ortho*-dihydroxybenzene of catechins (**Figure 1A**) (*11*), such as theaflavin (**1**), theaflavin-3-monogallate (**2**), theaflavine-3′-monogallate (**3**), and theaflavin-3,3′-digallate (**4**) (**Figure 1B**) (*11*, *30*), which are formed by oxidative coupling of the B-ring (3′,4′,5′-trihydroxyl) of one EGC or EGCG and the B-ring (3′,4′-dihydroxyl) of one EC (epicatechin) or ECG (epicatechin gallate). The theaflavate derivatives, theaflavates A (**5**) and B (**6**), are formed by condensation of the gallate ester group of ECG and the B-ring of ECG or EC (**Figure 1C**) (*31*). A new pigment, theadibenzotropolone A, recently characterized in black tea (*22*), is formed by oxidative coupling of the gallate ester group of theaflavin-3-monogallate and the B-ring of one EC (**Figure 1D**). The formation of a benzotropolone nucleus by oxidative coupling between 3,4,5-trihydroxylbenzene and 3,4-dihydroxylbenzene groups implies a loss of CH_4O, and therefore, 32 Da (*11*). For example, theaflavin (564 Da) is formed after oxidative reaction between one EC (290 Da) and one EGC (306 Da) (564 Da = 290 + 306 − 32). In the following paragraphs, compounds named "Theaflavin-type" suggest a loss of 32 Da by formation of a benzotropolone nucleus after oxidative condensation between a 3,4-dihydroxybenzene and a 3,4,5-trihydroxybenzene. Theasinensins and proanthocyanidins are also dimers of catechins in which monomers are typically linked through C6′−C6′ interflavonoid bonds for the "Theasinensins-type" (**Figure 1E**) and C4−C8 interflavanoid bonds for "Proanthocyanidin B-type" (**Figure 1F**). In the following paragraphs, compounds named "Theasinensin-type" or "Proanthocyanidin B-type" suggest a loss of 2 Da by formation of a single bond between two monomeric units of catechins. In our effort to understand TR structure, known TF standard molecules (**1**−**7**) were analyzed, and molecular ions and ions due to eventual in-source fragmentation are studied. Spectra of chromatographic fractions of black tea are compared to those of standard molecules. This comparison will allow us not only to formally identify the standard molecules in black tea fractions but also to propose some structures for TR-like molecules.

Spectra of Standard TF Molecule. Two types of ionization occur depending on the structure of the molecule. **Figure 2A** shows the spectrum of theaflavin (**1**) (MW 564 Da). Theaflavin has been ionized by protonation and formation of adduct ions, classically described for flavan-3-ols and proanthocyanidin derivatives by MALDI-TOF analysis (*32*−*36*), with formation of ions M + H^+ (*m*/*z* 565), M + Na^+ (*m*/*z* 587) and M + K^+

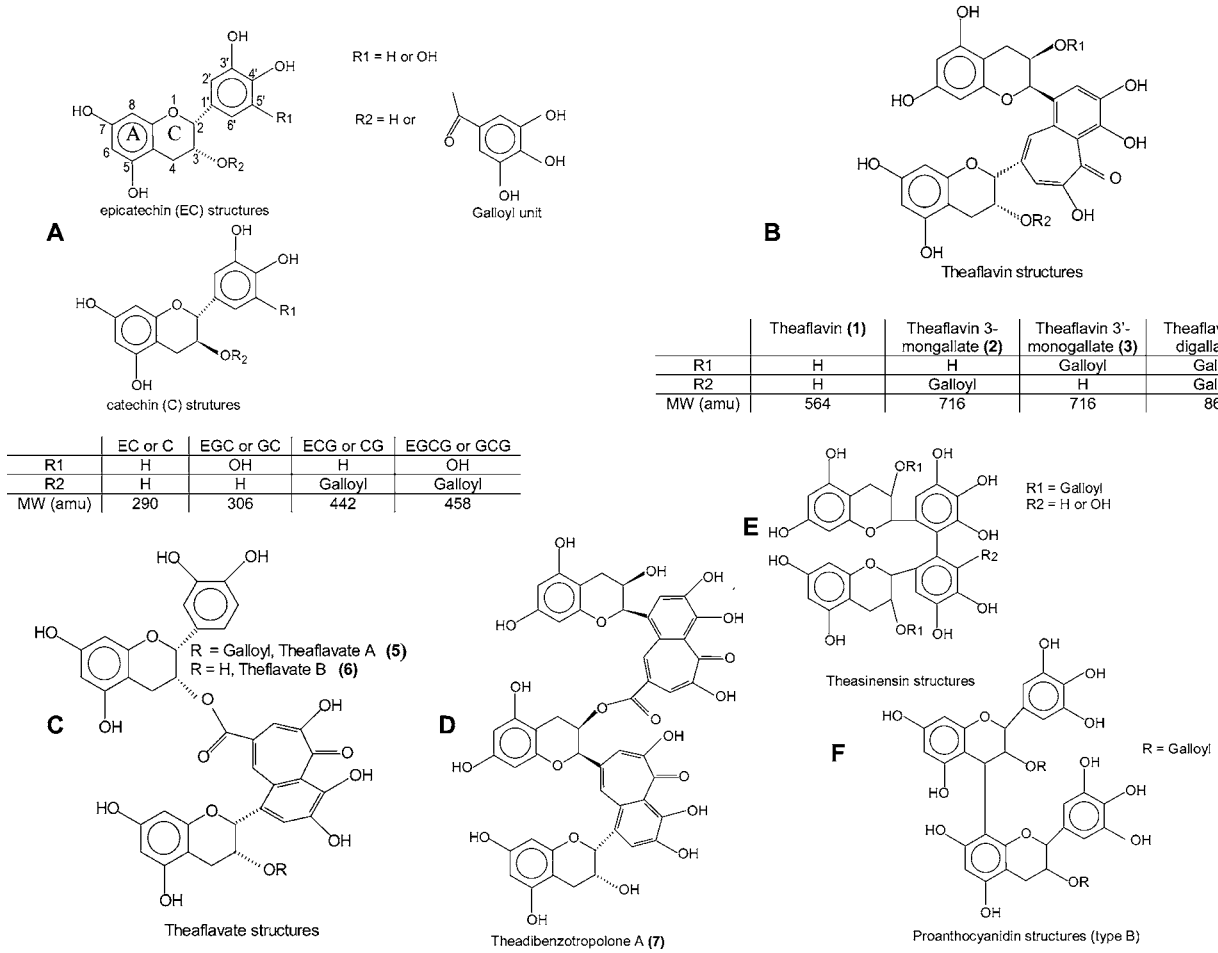

Figure 1. Structures of major components in tea.

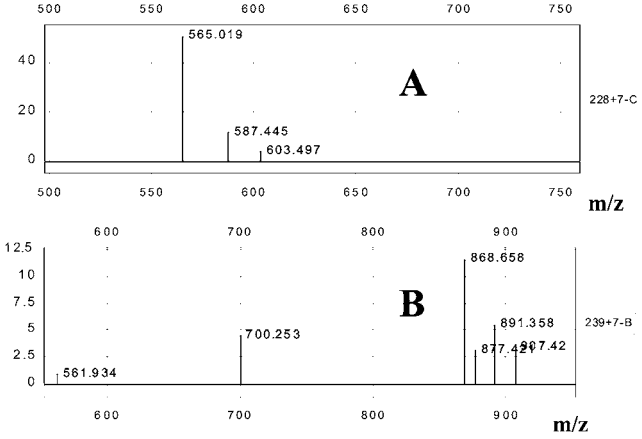

Figure 2. **A**: Spectrum of theaflavin (**1**) (reagent, 2,4,6-trihydroxyacetophenone, 10 mg/mL; solvent, methanol/water (7:3, v/v); theaflavin concentration, 0.2 g/L; laser energy, 228; sensitivity, 7). **B**: Spectrum of theaflavin-3,3′-digallate (**2**) (reagent, 2,4,6-trihydroxyacetophenone, 10 mg/mL; solvent, methanol/water (7:3, v/v); theaflavin-3,3′-digallate concentration, 0.2 g/L; laser energy, 239; sensitivity, 7).

(*m*/*z* 603). **Figure 2B** shows the spectrum of theaflavin-3,3′-digallate (**4**) (MW 868 Da). It is ionized with formation of ions $M + H^+$ (*m*/*z* 869), $M + Na^+$ (*m*/*z* 891) and $M + K^+$ (*m*/*z* 907). Fragmentation also occurs. During increased laser intensity, two types of fragment ions occur. The production of these fragment ions in the mass analysis of TF derivatives using MALDI-TOF has not been described prior to this report. The fragment ion at *m*/*z* 700 (699 + 1) is formed after cleavage of the ester functions as described in **Figure 3A**, and the loss of 169 Da corresponds to the loss of one gallate unit (170 Da = gallic acid). A proton is retained by the largest radical and not by the gallate loss. Fragment ion 562 is formed after a Retro-Diels−Alder (RDA) rearrangement of the *m*/*z* 700 fragment ion with loss of a neutral molecule of 138 Da (**Figure 3B**). This RDA rearrangement has been described for liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (*27*) and electron impact-mass spectrometry (*37*) for catechins, and liquid chromatography/electrospray ionization-mass spectrometry for proanthocyanidins (*38*). For these molecules, a proton is retained by the "ring A" part of molecules leading to the formation of an ion at *m*/*z* 139 (138 + H^+) for catechins and at various *m*/*z* for proanthocyanidins, depending on the degree of polymerization. For TF derivatives described in this paper, a proton is retained by the benzotropolone part ("ring B" part) presenting a larger aromaticity than the "ring A" part. Formation of adduct ions (by H^+, Na^+, and K^+), and fragmentation also occurs for theaflavin-3-monogallate (**2**), theaflavin-3′-monogallate (**3**), theaflavate A (**5**), theaflavate B (**6**), and theadibenzotropolone A (**7**). The *m*/*z* values of formed ions are listed in **Table 1**. Compounds **2**, **3**, and **5** present a loss of 169 Da as described in **Figure 3A** for theaflavin-3,3′-digallate because they are esters of gallic acid like theaflavin-3,3′-digallate (**4**). For **7**, the cleavage of ester function leads to a fragment ion at *m*/*z* 548, corresponding to a loss of 427 Da (**Figure 3A**). Compounds **5** and **6** present similar structures to **7** because they are esters of an acid presenting a benzotropolone structure, but the eventual cleavage of this ester function leads

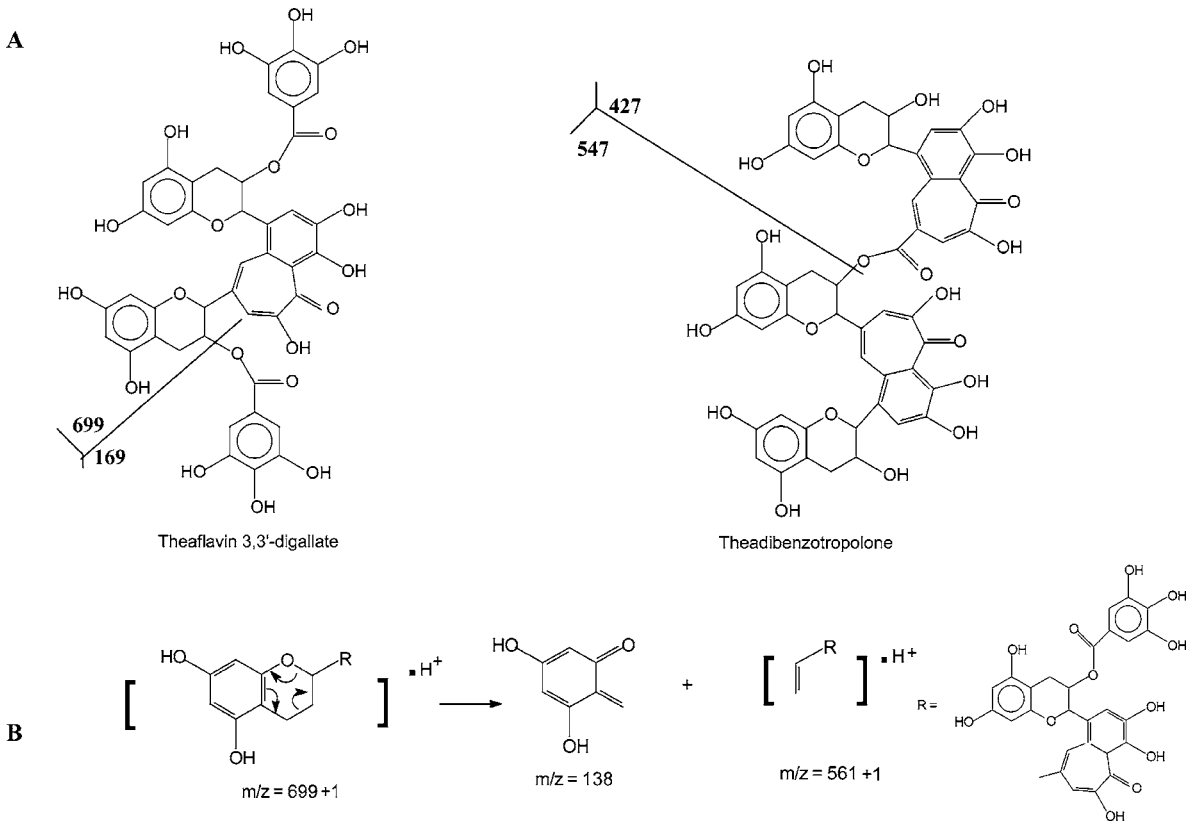

Figure 3. Fragmentation mechanisms: **A**, cleavage of ester functions; **B**, retro-Diels–Alder.

Table 1

compound	adduct H^+ (*m*/*z*)	adduct Na^+ (*m*/*z*)	adduct K^+ (*m*/*z*)	ion fragment (*m*/*z*)	loss (*m*/*z*)
theaflavin **(1)**	565	587	603		
theaflavin-3-monogallate **(2)** and theaflavin-3′-monogallate **(3)**	717	739	755	548	169
theaflavin-3,3′-digallate **(4)**	869	891	907	700, 562	169
					169 + 138
theaflavate A **(5)**	853	875	891	700	153
				683	169
theaflavate B **(6)**	701	723	739		
theadibenzotropolone **(7)**	975	997		548	427

to ion fragments at *m*/*z* lower than 500 and are not taken into account in this paper.

In conclusion, standard molecule analysis by MALDI-TOF with DE shows TF derivatives are fragmented by cleavage of the ester functions with formation of fragment ions corresponding to a loss of 169 and/or 153 Da when gallate is present in the molecule and 427 Da when it is a theaflavate type trimer (**Table 1**). RDA rearrangement is possible after fragmentation and loss of ester part and leads to a stable fragment ion containing the benzotropolone structure. These characteristic ions allow us to positively identify TF standard molecules in black tea fractions and propose some structures for TR.

Fraction Analysis. The 80% acetone extract of black tea was concentrated to dryness under reduced pressure, and the residue was dissolved in water and partitioned with either chloroform, ethyl acetate, or *n*-butanol. The ethyl acetate fraction was subjected to Sephadex LH-20 column eluted by an acetone/water solvent system (30%−60%) to give 14 fractions. These 14 fractions and the *n*-butanol fraction were directly analyzed by MALDI-TOF. **Figure 4** shows the spectra obtained from two fractions, fraction 13 (**Figure 4A**) and the *n*-butanol fraction (**Figure 4B**).

Standard Molecules. TF standards were found in fractions according to their MW and polarity (**Figure 4A**). Theaflavin is contained in fractions 7−11 with a characteristic peak detected at *m*/*z* 565 ($M + H^+$), theaflavate B in fraction 12 with a characteristic peak detected at *m*/*z* 701 ($M + H^+$), theaflavin monogallates in fractions 11−13 with a characteristic peak at *m*/*z* 717 ($M + H^+$), theaflavate A in fraction 13 with characteristic peaks at *m*/*z* 853 ($M + H^+$) and *m*/*z* 700 (($F + H^+$) fragment ion formed after cleavage of the ester function and loss of 153), and theaflavin digallate in fractions 13 and 14 with characteristic peaks at *m*/*z* 869 and 891 ($M + H^+$ and $M + Na^+$, respectively), at *m*/*z* 700 ($F + H^+$), fragment ion formed after cleavage of the ester function and loss of 169 Da), and at *m*/*z* 562 ($F + H+$), fragment ion formed after RDA reaction and retention of proton by the benzotropolone part (**Figure 3B**). These spectral analyses prove the ability of MALDI-TOF to directly analyze black tea fractions and detect standard TFs by their specific pseudomolecular and fragment ions. This method can, therefore, be applied to the detection of unidentified polyphenolic compounds (such as TRs) in black tea fractions by comparing the ionization characteristics with those of standard TFs.

Polymers. The spectrum of the *n*-butanol fraction (**Figure 4B**) shows many peaks at *m*/*z* higher than 1000 that do not correspond to standard molecules analyzed. Their structures are proposed according to the pseudomolecular and fragment ions detected. Two intense and large peaks at *m*/*z* 594 ($F + H^+$) and 593 appeared in the spectrum of the *n*-butanol fraction (**Figure 4B$_1$**). Moreover, standard spectra show that 153, 169, and 427 Da mass losses are encountered during analysis. So, when 427 and 153 Da are added to 593 Da ($F+ H^+$), the molecular weight of the potential compound is 1172 Da. Spectrum of fraction B1 (**Figure 4B$_2$**) shows a peak at *m*/*z* 1173

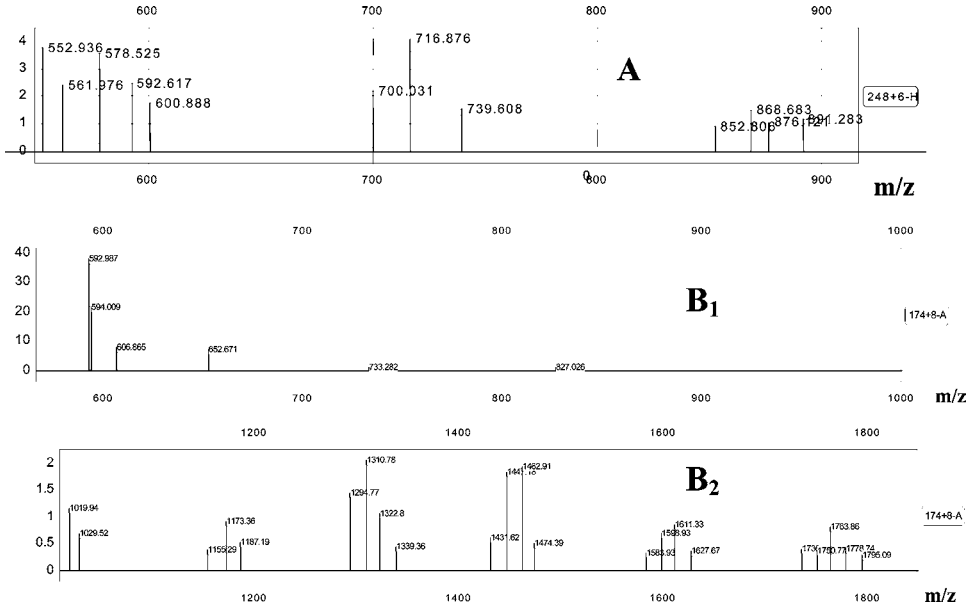

Figure 4. **A**: Spectrum of fraction 13 (reagent, 2,4,6-trihydroxyacetophenone, 10 mg/mL; solvent, methanol/water (7:3, v/v); concentration, 0.82 g/L; laser energy, 248; sensitivity, 6). **B_1** and **B_2**: Spectrum of *n*-butanol fraction (reagent CHCA, saturated concentration; solvent, methanol/water (7:3, v/v); concentration, 30 g/L; laser energy, 174; sensitivity, 8. TF, theaflavin types; PA, proanthocyanidin-types).

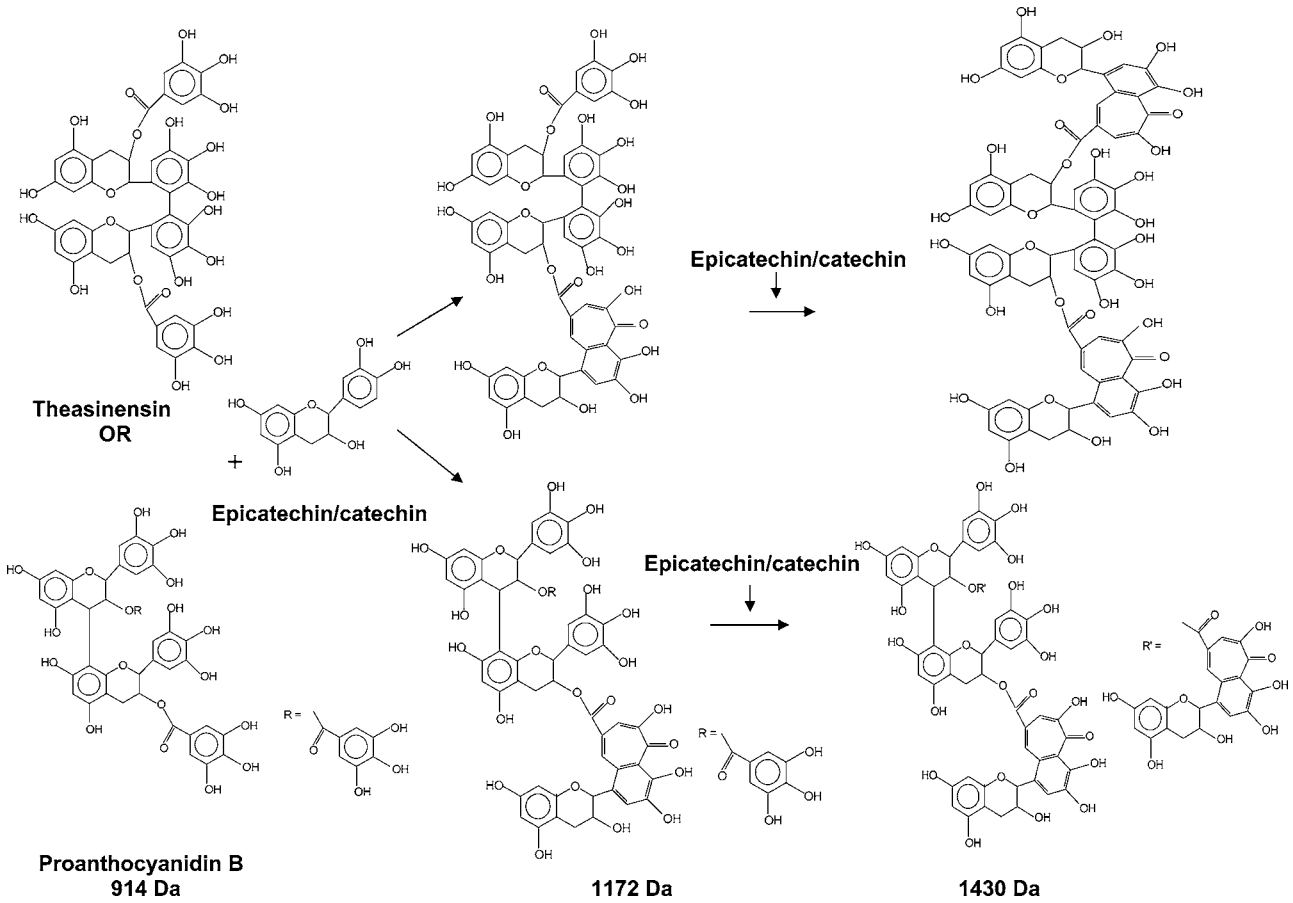

Figure 5. Proposed structures and formation mechanism for some thearubigins.

Da. This compound would be a diester of an acid containing a benzotropolone nucleus (loss of 427 Da) and a galloyl group (loss of 153 Da). These two esters are cleaved during MALDI-TOF analysis with successive loss of 153 Da to form fragment ion at *m*/*z* 1020 Da and loss of 427 Da to form fragment ion *m*/*z* 593 Da. Two different structures can be proposed for these compounds (**Figure 5**). They can derive from dimers of catechins, such as proanthocyanidin (EGCG-4-α-EGCG or Prodelphinidin B2) (*32*−*34*) or theasinensin (*10*). The compound, which has a molecular weight 1172, can be formed from condensation of one gallate ester group of one dimer of catechins and the B-ring of EC/C (914 + 290 − 32 Da) to give a benzotropolone skeleton with a mass loss of 32 Da (CH_4O) (*11*) (**Figure 5**). If both gallate ester groups of one dimer have been

condensed with B-rings of two EC/C, the molecular mass of the formed compound is *m*/*z* 1430 (914 + 290 + 290 − 32 − 32 Da). The spectrum of the *n*-butanol fraction shows a peak at *m*/*z* 1431. The structure of *m*/*z* 1430 compound is proposed in **Figure 5**. The *n*-butanol fraction spectrum also shows compounds with molecular masses different then that of *m*/*z* 1430, from 16 and 32 Da, *m*/*z* 1447 and 1463, respectively. A previous study introduces the hypothesis that some TRs can be formed after condensation of one EGC and one EGCG (*16*, *17*). These results suggest that these theaflavin-type compounds can be generated from the condensation between a gallate ester group of theasinensin or proanthocyanidin and one EC/C and one EGC/GC for compound *m*/*z* 1446 and two EGC for compound *m*/*z* 1462. These compounds therefore present two free OH groups not esterified by gallic acid. If one galloyl unit (152 Da) is added to *m*/*z* 1446, the molecular weight of the potential compound is 1598 Da with the *n*-butanol fraction spectrum presenting a peak at *m*/*z* 1599. If a second galloyl unit is added to 1598 Da, the molecular weight of the potential compound is 1750 Da with the *n*-butanol fraction spectrum presenting a peak at *m*/*z* 1751. A part of TRs, therefore, consist of a mixture of molecules coming from the condensation of 3,4,5-trihydroxyl of gallates of a dimer (such as theasinensin or proanthocyanidin) and catechin derivatives ECG/CG, EC/C, EGC/CG, or EGCG/CGC.

The current study shows that MALDI-TOF, associated with DE, is a powerful technique to analyze standards of TFs and to give ionization patterns for these molecules, in particular by adduct ion and fragmentation of ester functions. These characteristics allow direct detection of TF standards in black tea extract and indicate TR structures. Some TRs are polymers of catechins in which the 3-OH group is more and less esterified by gallic acid. Others are derivatives of dimers of catechins for which the gallate part has been condensed with "B-ring" catechins. This study is one step in the comprehension of TR structures. Further studies should use this information to synthesis TR standards before their analysis by MALDI-TOF-DE, which will give interesting information about the ionization patterns of these molecules to confirm their structures and to detect them directly in black tea fractions.

LITERATURE CITED

(1) Higdon, J. V.; Frei, B. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit. Rev. Food Sci. Nutr*. **2003**, *43* (*1*), 89−143.

(2) Yang, C. S.; Landau, J. M. Effects of tea consumption on nutrition and health. *J. Nutr*. **2000**, *130*, 2409−2412.

(3) Frei, B.; Higdon, J. V. Antioxidant activity of tea polyphenols in vivo: Evidence from animal studies. *J. Nutr*. **2003**, *133*, 3275s−3284s.

(4) Yang, C. S.; Maliakal, P.; Meng, X. Inhibition of carcinogenesis by tea. *Annu. Rev. Pharmacol. Toxicol*. **2002**, *42*, 25−54.

(5) Lambert, J. D.; Yang, C. S. Mechanisms of cancer prevention by tea constituents. *J. Nutr*. **2003**, *133*, 3262s−3267s.

(6) Vita, J. A. Tea consumption and cardiovascular disease: Effects on endothelial function. *J. Nutr*. **2003**, *133*, 3293s−3297s.

(7) Lin, Y. L.; Tsai, S. H.; Lin-Shiau, S. Y.; Ho, C.-T.; Lin, J. K. Theaflavin-3,3′-digallate from black tea blocks the nitric oxide synthase by down-regulating the activation of NF-κB in macrophages. *Eur. J. Pharmacol*. **1999**, *367*, 379−388.

(8) Hong, J.; Smith, T.; Ho, C.-T.; August, D. A.; Yang, C. S. Effects of purified green and black tea polyphenols on cyclooxygenase- and lipoxygenase-dependent metabolism of arachidonic acid in human colon mucosa and colon tumor tissues. *Biochem. Pharmacol*. **2001**, *62*, 1175−1183.

(9) Satoh, E.; Ishii, T.; Shimizu, Y.; Sawamura, S.-I.; Nishimura, M. Black tea extract, thearubigin fraction, counteract the effects of botulinum neurotoxins in mice. *Brit. J. Pharm*. **2001**, *132*, 797−798.

(10) Harbowy, M. E.; Balentine, D. A. Tea chemistry. *Crit. Rev. Plant Sci*. **1997**, *16 (5)*, 415−480.

(11) Geissman, T. A. *Chemistry of Flavonoid Compounds*. Pergamon Press: Oxford, UK, 1962; pp 468−512.

(12) Roberts, E. A. H. The phenolic substances of manufactured tea. II − Their origin as enzymic oxidation products in fermentation. *J. Sci. Food Agric*. **1958**, *9*, 212−216.

(13) Bailey, R. G.; Nursten, H. E. Comparative study of the reversed-phase high-performance liquid chromatography of black tea liquors with special reference to the thearubigins. *J. Chromatogr*. **1991**, *542*, 115−128.

(14) Ozawa, T.; Kataoka, M.; Morikawa, K.; Negiski, O. Elucidation of the partial structure of polymeric thearubigins from black tea by chemical degradation. *Biosci. Biotechnol. Biochem*. **1996**, *60*, 2023−2027.

(15) Robertson, A. Effects of catechin concentration on the formation of black tea polyphenols during in-vitro oxidation. *Phytochemistry* **1983**, *22(4)*, 897−903.

(16) Robertson, A. Effects of physical and chemical conditions on the in-vitro oxidation of tea leaf catechins. *Phytochemistry* **1983**, *22(4)*, 889−896.

(17) Robertson, A.; Bendall, D. S. Production and HPLC analysis of black tea theaflavins and thearubigins during in-vitro oxidation. *Phytochemistry* **1983**, *22 (4)*, 883−887.

(18) Finger, A. In vitro studies on the effect of polyphenol oxidase and peroxidase on the formation of polyphenolic black tea constituent. *J. Sci. Food Agric*. **1994**, *66*, 293−305.

(19) Opie, S. C.; Clifford, M. N.; Robertson, A. The formation of thearubigin-like substances by in-vitro polyphenol oxidase-mediated fermentation of individual flavan-3-ols. *J. Sci. Food Agric*. **1995**, *67*, 501−505.

(20) Opie, S. C.; Clifford, M. N.; Robertson, A. The role of (−)-epicatechin and polyphenol oxidase in the coupled oxidative breakdown of theaflavins. *J. Sci. Food Agric*. **1993**, *63*, 435−438.

(21) Subramanian, N.; Venkatesh, P.; Ganguli, S.; Sinkar, V. P. Role of polyphenol oxidase and peroxidase in the generation of black tea theaflavins. *J. Agric. Food Chem*. **1999**, *47*, 2571−2578.

(22) Sang, S.; Tian, S.; Meng, X.; Stark, R. E.; Rosen, R. T.; Yang, C. S.; Ho, C.-T. Theadibenzotropolone A, a new pigment from enzymatic oxidation of (−)-epicatechin and (−)-epigallocatechin gallate and characterized from black tea using LC/MS/MS. *Tetrahedron Lett*. **2002**, *43*, 7129−7133.

(23) Sporns, P.; Wang, J. Exploring new frontiers in food analysis using MALDI-TOF. *Food Res. Int*. **1998**, *31*, 181−189.

(24) Karas, M.; Bachmann, D.; Bahr U.; Hillenkamp; F. Matrix-assisted ultraviolet laser desorption of nonvolatile compounds. *Int. J. Mass Spectrom. Ion Processes* **1987**, *78*, 53−68.

(25) Spengler, B.; Kirsch, D.; Kaufmann, R. Metastable decay of peptides and proteins in matrix-assited laser-desorption mass spectrometry. *Rapid Commun. Mass Spectrom*. **1991**, *5*, 198−202.

(26) Brown, R. S.; Lennon, J. J. Sequence-specific fragmentation of matrix-assisted laser-desorbed protein/peptide ions. *Anal. Chem*. **1995**, *67*, 3990−3999.

(27) Zeeb, D. J.; Nelson, B. C.; Albert, K.; Dalluge, J. J. Separation and identification of twelve catechins in tea using liquid chromtography/atmospheric pressure chemical ionization-mass spectrometry. *Anal. Chem*. **2000**, *72*, 5020−5026.

(28) Sang, S.; Lamber, J. D.; Tian, S. Y.; Hong, J.; Hou, Z.; Rya, J. H.; Stark, R. E.; Rosen, R. T.; Huang, M. T.; Yang, C. S.; Ho, C.-T. Enzymatic synthesis of tea theaflavin derivatives and their antiinflammatory and cytotoxic activities. *Bioorg. Med. Chem*. **2004**, *12*, 459−467.

(29) Frisson-Norrie, S.; Sporns, P. Identification and quantification of flavonol glycosides in almond seedcoats using Maldi-TOF MS. *J. Agric. Food Chem*. **2002**, *50*, 2782−2787.

(30) Runeckles, V. C.; Tso, T. C. *Recent Advances in Phytochemistry*; Academic Press: New York, 1972; Vol. 5, 247−316.

(31) Wan, X.; Nursten, H. E.; Cai, Y.; Davis, A. L.; Wilkins, J. P. G.; Davies, A. P.A New type of tea pigment − from the chemical oxidation of epicatechin gallate and isolated from tea. *J. Sci. Food Agric*. **1997**, *74*, 401−408.

(32) Ohnishi-Kameyama, M.; Yanagida, A.; Kanda, T.; Nagat, T. Identification of catechin oligomers from apple (*Malus pumila* cv. fuji) in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and fast-atom bombardment mass spectrometry. *Rapid. Commun. Mass Spectrom*. **1997**, *11*, 31−36.

(33) Behrens, A.; Maie, N.; Knicker, H.; Kögel-Knabner, I. MALDI-TOF mass spectrometry and PSD fragmentation as means for the analysis of condensed tannins in plant leaves and needles. *Phytochemistry* **2003**, *62*, 1159−1170.

(34) Takahata, Y.; Ohnishi-Kameyama, M.; Furuta, S.; Takahashi, M.; Suda. I. Highly polymerized procyanidins in brown soybean seed coat with a high radical-scavenging activity. *J. Agric. Food Chem*. **2001**, *49*, 5843−5847.

(35) Krueger, C. G.; Dopke, N. C.; Treichel, P. M.; Folts, J.; Reed, J. D. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of polygalloyl polyflavan-3-ols in grape seed extract. *J. Agric. Food Chem*. **2000**, *48*, 1663−1667.

(36) Yang, Y.; Chien. M. Characterization of grape procyanidins using high-performance liquid chromatography/mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Agric. Food Chem*. **2000**, *48*, 3990−3996.

(37) Miketova, P.; Schram, K. H.; Whitney, J. L.; Kerns, E. H.; Valcic, S.; Timmermann B. N.; Volk. K. J. Mass spectrometry of selected components of biological interest in green tea extracts. *J. Nat. Prod*. **1998**, *61*, 461−467.

(38) Zywicki, B.; Reemtsma, T.; Jekel, M. Analysis of commercial vegetable tanning agents by reversed-phase liquid chromatography-electrospray ionization-tandem mass spectrometry and its application to wastewater. *J. Chromatogr. A.* **2002**, *970 (1−2)*, 191−200.

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