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Identification of Novel Homologous Series of Polyhydroxylated Theasinensins and Theanaphthoquinones in the SII Fraction of Black Tea Thearubigins Using ESI/HPLC Tandem Mass Spectrometry

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Supporting Information

ABSTRACT: Thearubigins are the most abundant phenolic pigments found in black tea, produced by enzymatic oxidation of green tea flavan-3-ols in tea fermentation of until recently unknown composition. In this study electrospray ionization tandem LC-MS^n experiments have been applied for the characterization of crude thearubigins isolated from black tea not exceeding 1000 Da. The aim of this study is to confirm the oxidative cascade hypothesis of tea fermentation. The data revealed the presence of two novel classes of compounds in thearubigin fractions. The first class of compounds revealed the presence of polyhydroxylated dimers of the theanaphthaquinone and theasinensin C structures, which were consistent with the polyhydroxylation hypothesis previously formulated. Furthermore, new classes of peroxo-/epoxy- compounds in the series of theasinensin A were identified, thus indicating the presence of H_2O_2 and its important contribution as a nucleophile in the tea fermentation process.

KEYWORDS: *thearubigins, oxidative cascade hypothesis, black tea, polyphenols,, tandem mass spectrometry*

INTRODUCTION

Black tea is globally the most consumed beverage after water. The average tea consumption per person per year worldwide stands at 0.5 kg, which accounts for the consumption of an estimated 150 L per year or 500 mL per person per day.[1] In 2012, the United Nations Food Agency and Agricultural Organization (FAO) reported that tea production reached 4.05 million tons, comprising 20% green tea and 2% oolong tea with the remaining 78% black tea.[2] Black tea is mainly produced by a systematic manufacturing process from the fresh green leaves of *Camellia sinensis* or *Camellia assamica* plants, which are rich in flavan-3-ols, by a so-called fermentation process. Tea fermentation constitutes an enzymatically driven oxidative process in the absence of microorganisms. Both polyphenol oxidase (TPPO) and peroxidase enzymes (POD) were found to be responsible for the oxidation of flavan-3-ols acting as the main substrates for the various oxidase enzymes in the green tea leaf, that is, epigallocatechin gallate (**5**), epicatechin gallate (**6**), epigallocatechin (**4**), and epicatechin (**2**)[3] (Figure 1). The substrates are oxidized and extensively transformed into novel dimeric, oligomeric, and polymeric compounds in a heterogeneous mixture known as thearubigins. Thearubigins (TRs) were previously identified to be the most abundant group of phenolic pigments in black tea, thereby accounting for an estimated 75% of the solids in a typical black tea infusion[4,5] and contributing to a considerable extent to its color, taste, and quality. However, the structures of thearubigin components remained poorly characterized despite numerous attempts to resolve their structure by several research groups over the past 50 years. The failure to obtain any meaningful data on TRs can be traced to the complexity of the material and the limitations of analytical techniques available at the time. Identification and structure elucidation of the individual constituents of thearubigins is important for understanding those components contributing to many physicochemical properties of black tea that stand behind the astringent and bitter or desirable taste,[6] the dark color,[7] and other properties, thus facilitating the manufacturing of tea-based products. On the other hand, one can ascertain the identification of chemical compounds, which are responsible for the beneficial health effects supposedly associated with the consumption of black tea.[8]

When analyzing black tea, which is classified as a complex mixture, Kuhnert et al. adopted a novel data interpretation strategy based on ultrahigh-resolution mass spectrometry coupled to targeted tandem mass spectrometry. From ESI-FT-ICR-MS data we employed van Krevelen and Kendrick analysis as a powerful graphical tool for understanding the complexity of the thearubigin constituents present.[9] The data revealed that a typical TR fraction contains around 30000 individual constituents. The van Krevelen analysis, known as elemental ratio analysis, allowed tentative classification of TR components, with 90% of the constituents being identified as polyphenols.[10,11] Kendrick analysis from FT-ICR-MS data displays information with respect to mass defects present in a class of compounds.[12] Mass increments were identified for homologous series of compounds and structures of homologous series of polyhydroxylated flavan-3-ol dimers postulated.[13] This petrolomic strategy was adopted for the first time in food chemistry, allowing a rational interpretation of complex mixtures obtained in food processing.[14,15] The data generated revealed the presence of different mass increments of oxygen

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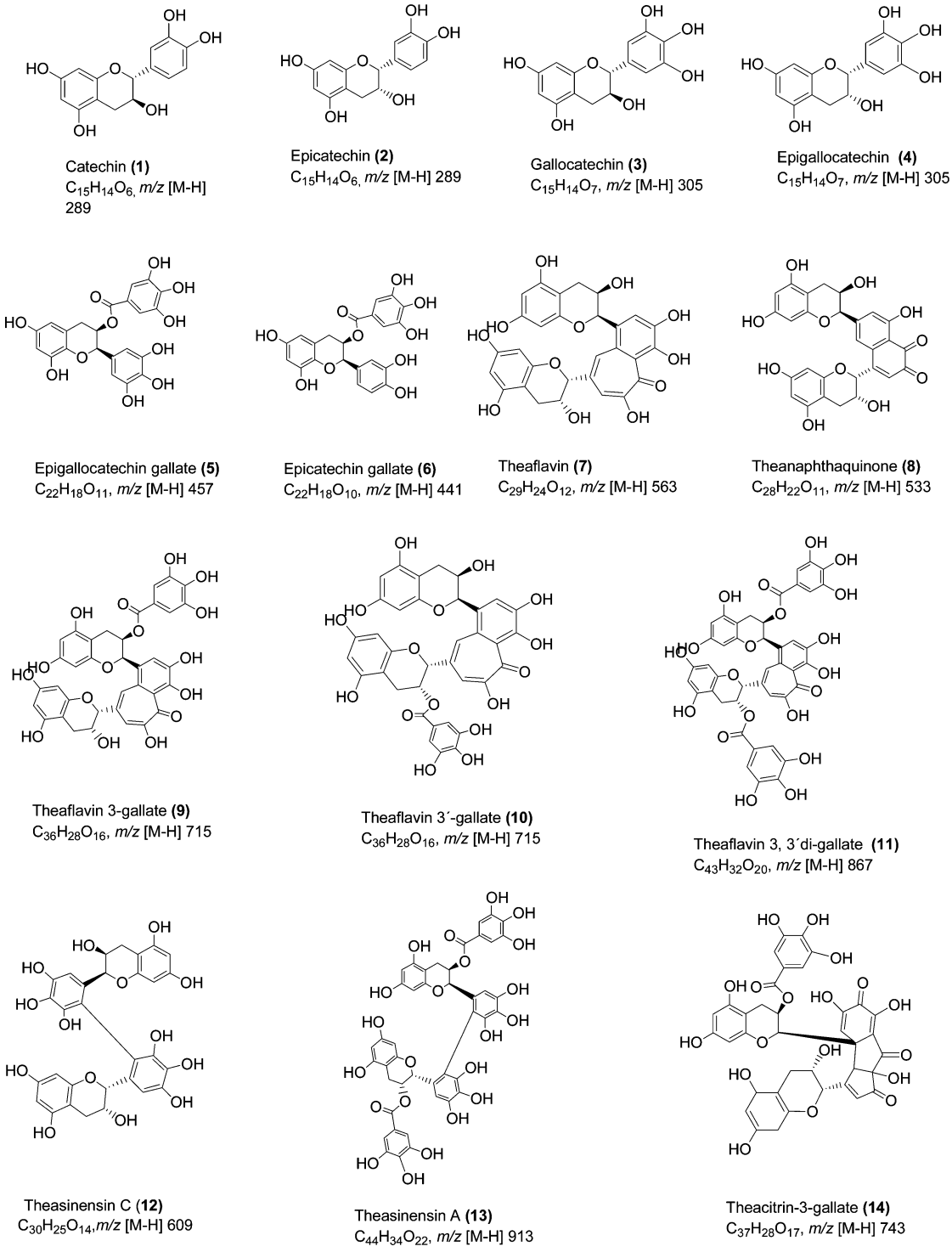

Figure 1. Structures of catechins and their formal dimers previously identified in black tea.

(O), hydrogen (H_2), gallates ($C_7H_5O_4$), and catechins in various oxygenation degrees acting as main building blocks in black tea chemistry. According to the data generated, oxygen insertion was observed as mass increment in the largest number.[16] However, the type and nature of the compounds were still unknown. Later, on the basis of initial ESI-FTICR-MS data[17,18] from targeted tandem LC-MS experiments, the data revealed that a significant fraction of thearubigins consists of polyhydroxylated derivatives of catechin dimers (a stepwise increase of oxygen increments within the dimeric structures), the so-called "homologous series".[9] On the basis of the experimental data, the oxidative cascade hypothesis was proposed. This hypothesis proposes the formation of the *o*-quinones of different dimeric and oligomeric structures in the oxidation process, where these could be followed by nucleophilic addition of water in the fermentation process, leading to polyhydroxylated structures, and this continues until all aromatic hydrogens are replaced by OH functionalities, thus increasing the number of the components within the thearubigin fractions. Previous investigations carried out on

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the oxidative cascade hypothesis revealed the presence of homologous series of theaflavins (TFs), their gallate esters theaflavin mono- and digallates, and theacitrin (TC) structures.[16,17] Kuhnert et al. additionally showed that a significant portion of these compounds are in redox equilibrium with their quinone counterparts.[17] MALDI-MS experiments on TRs[18] and detailed tandem MS investigations by Yassin et al. additionally showed that trimers and teramers of flavan-3-ols are major products of oxidative tea fermentation.[18]

The aim of this study is to further confirm the second level of the oxidative cascade hypothesis and to structurally elucidate the novel thearubigin components. For this investigation electrospray ionization tandem mass spectrometry (ESI-LC-ion trap-MS) was employed for characterizing thearubigins in homologous series reaching m/z 1000. A particular emphasis was put on derivatives of theasinensins (TSs, also referred to as bisflavanones) and theanaphthaquinones (TNQs), the two dimeric catechin derivatives so far not investigated. The experimental strategy used was based on generating extracted ion chromatograms EIC at the mass of the parent ion and their corresponding hydroxylated derivatives, where the signals are strong enough to obtain MS^2 and MS^3 spectra. In this approach, the different m/z signals of the parent ion of theanaphthaquinone and theasinensin C dimeric structures and their hydroxylated components were obtained in the homologous series, where several regioisomers were detected at distinct retention times, increasing the structural variety of the newly identified thearubigin compounds.

Furthermore, interestingly a new series of compounds could be also identified for the first time revealing the presence of H_2O_2 as a contributor in the fermentation process.[19] Previous studies done by Sinkar et al. confirmed the presence of H_2O_2 and its important role in the oxidation process leading to the formation of thearubigins in the presence of peroxidase enzyme.[20] In this investigation, by focusing on the ESI-LC-tandem mass MS^n measurements, the homologous series of dimeric structures theasinensin C and theanaphthaquinone are obtained, and a new series containing epoxide derivatives of theasinensin A that has not been previously investigated are reported.

MATERIALS AND METHODS

Chemicals and Reagents. All chemicals and reagents were purchased from Sigma-Aldrich. Black tea samples of Ceylon, Assam, and Indian tea varieties were purchased from a local supermarket and provided from the Unilever Excellence Center.

Preparation of Thearubigins. Freshly ground black tea leaves (8 g) were added to 150 mL of freshly boiled water and kept for 10 min in a Thermos flask, which was inverted every 30 s. The flask contents were filtered through a Whatman no. 4 filter paper to remove the leaves, and the remaining brew was allowed to cool to room temperature. Caffeine sufficient to achieve 20 mM was added to the brew, stirred to ensure dissolution, allowed to stand at 4 °C for 2 h, and centrifuged at 23300*g* for 20 min. The resulting precipitate was recovered and suspended in boiling water and partitioned against aliquots of ethyl acetate (40 mL) until no further color was extracted (usually five times).

The ethyl acetate supernatant was removed and evaporated to dryness under nitrogen below 35 °C, and the residue (TF fraction) was recovered in 10 mL of distilled water. The aqueous phase was partitioned at 80 °C against 2 volumes of chloroform, and the decaffeinated liquid was stored overnight at −80 °C and freeze-dried. The freeze-dried material (TR fraction) was stored at −20 °C until required and reconstituted as required for the analysis. The yield of thearubigins in this method was mainly 10% and obtained as orange to light brown fluffy powders.

LC-MS^n Method. The extracted thearubigin solutions were reconstituted to a 0.5 mg/mL concentration in 1:1 methanol/water, filtered through a 0.45 μm HPLC filter, and analyzed by LC-MS method using Aligent 1100 series LC equipment and a DAD detector with a light pipe flow cell (recording at 400 and 245 nm and scanning from 200 to 600 nm). This was coupled to an ion trap mass spectrometer fitted with an ESI source (Bruker Daltonics HCT Ultra) operating in the negative ion Auto MS^n mode to obtain fragment ion m/z. Separations were achieved on a Polaris 5-C18-A column (length, 250 mm; diameter, 3 mm; particle size, 5 μm) with a step gradient elution employing acetonitrile (MeCN) and water containing 0.005% formic acid, as follows: 8% MeCN from 0 to 50 min, then changing to 31% MeCN for 10 min, then changing to 25% MeCN for a further 5 min. The column eluent was first directed to the UV detector and then to the ESI interface operating with a capillary voltage of 1 V, and fragmentation amplitude was set starting at 30% and ending at 200%. The capillary temperature was also set at 300 °C. Nitrogen gas was used here as nebulizing and drying gas at a flow rate of 10 L/min and a pressure of 10 psi, respectively.

High-Resolution LC-MS. High-resolution LC-MS in the negative ion mode was carried out using the same HPLC equipped with a MicrOTOF Focus mass spectrometer (Bruker Daltonics) fitted with an ESI source, and internal calibration was achieved with 10 mL of 0.1 M sodium formate solution injected through a six-port valve prior to each chromatographic run. Calibration was carried out using the enhanced quadratic calibration mode. It should be noted that in TOF calibration the intensities of the measured peaks have a significant influence on the magnitude of the mass error with high-intensity peaks, resulting in detector saturation displaying larger mass errors. Where necessary, this was avoided by using a more dilute sample. All MS measurements were carried out in the negative ion mode.

HPLC Analysis. The extracted thearubigins were analyzed by HPLC using an Agilent 1200 HPLC pump with a 5 μL loop, coupled to an Agilent 1100 autosampler and an Agilent 1100 DAD-UV−vis detector. Black tea extracts and thearubigin extracts were reconstituted at 5 mg/mL in 1:1 MeOH/H_2O and filtered through a 0.45 μm HPLC filter prior to injection of a volume of 3 μL. HPLC analysis used a Polaris 5-C18-A column (length, 250 mm; diameter, 3 mm; particle size, 5 μm) with a step gradient elution employing acetonitrile (MeCN) and water containing 0.005% formic acid, as follows: 8% MeCN from 0 to 50 min, then changing to 31% MeCN for 10 min, then changing to 25% MeCN for a further 5 min.

Data Analysis. Data were analyzed using Bruker Data Analysis 4.0 software. Micro TOF data were analyzed (TICs, EICs) after external enhanced quadratic calibration. Ion trap data were analyzed in terms of EICs, TICs, and neutral loss chromatograms (NLCs) using the implemented software routines.

RESULTS AND DISCUSSION

For further investigating the hydroxylation process of the dimeric components and confirming their presence within the oxidative cascade hypothesis in thearubigin fractions, ESI-LC-MS^n experiments were carried out on the homologous series of theasinensin C (**12**) and its gallate ester theasinensin A (**13**) (Figure1). These structures are formed via dimerization of two catechin derivatives. Additionally, a homologous series of theanaphthaquinones (**8**) (Figure 1) were investigated, which is formed via further oxidation of theaflavin. Both dimeric structures are likely implicated in the thearubigin formation.

LC−tandem MS experiments were performed on the selected m/z parent ions expected for these compounds, which are potentially the first member of the anticipated homologous series. Extracted ion chromatograms (EICs) were created from the m/z values of hypothetical members of the homologous series, and in case strong signals were observed at the corresponding m/z values, targeted fragmentation experi-

Table 1. Homologous Series for Theanaphthaquinone Confirming the Presence of the Hydroxylated Derivatives with Their Selected MS^2 and MS^3 Fragment Ions and Neutral Losses Observed[a]

	molecular formula	RT[b] (min)	LC-MS [M − H] (m/z)	fragment ion LC-MS^2 (m/z)	neutral loss δM(S1 − S2)[c] (Da)	fragment ion LC-MS^3 (m/z)
8	$C_{28}H_{21}O_{11}$	47.9	533	**514.8 (100%)**, 464.6 (55.9%), 396.6 (31.6%)	18.2, 69, 137	304.7 (100%), 376.8 (40%)
8 + O_2	$C_{28}H_{21}O_{13}$	50.1	565.1	**544.9 (100%)**, 494.5 (40.5%)	20.7, 70.1	240.6 (100%)
8 + O_3	$C_{28}H_{21}O_{14}$	53.5	581.2	**512.5 (100%)**, 444.5 (33.1%)	69, 138	444.5 (100%), 376.5 (18%)
		43	581.3	**512.5 (100%)**, 444.5 (19%)	69, 137	444.5 (100%), 376.5 (25%)
		34.7	581.3	**512.5 (100%)**, 444.5 (20%)	69, 137	444.5 (100%)
		30.9	581.2	**512.5 (100%)**, 444.5 (31.4%)	69, 137	444.5 (100%), 376.4 (21.3%)
8 + O_4	$C_{28}H_{21}O_{15}$	48.4	597	**528.5 (100%)**	69	402.5 (100%)
8 + O_5	$C_{28}H_{21}O_{16}$	39	613.2	**544.5 (100%)**, 476.5 (5%)	69, 137	402.5 (100%)
		28.1	613.1	**544.5 (100%)**, 476.5 (5.2%)	69, 137	402.5 (100%)

[a]MS^2 fragment ions highlighted in bold are subjected for further fragmentation for MS^3 fragment ions. [b]RT, retention time (min). [c]δM(S1 − S2), mass loss (Da).

ments were carried out corresponding to MS^2 and MS^3 spectra. The presence of fragment ions with their neutral losses was located; therefore, the fragmentation pattern and its mechanism have been determined for the parent structure forming the first member of each homologous series. Later, oxygen mass increments were introduced, and different signals at m/z values corresponding to the majority of predicted hydroxylated derivatives were established. The interpretation here was based on the fragment ions and the neutral losses for the parent ions observed, where the mechanism of fragmentation has been previously established either from investigation of authentic reference compounds or from literature precedents.[16] The assumption for identifying the homologous series of different hydroxylated parent ions was based on their m/z values and their MS^2 fragment spectra observed. An identical fragmentation mechanism has to be obtained for the whole homologous series. For example, theaflavins (**7** shown in Figure 1) undergo fragmentation with a neutral loss of an enone of m/z 137 ($C_7H_6O_3$), which can be rationalized in terms of retro Diels-Alder fragmentation (RDA) at one of the benzopyran moieties.[21] It has been confirmed that the whole homologous series of this compound underwent the same fragmentation.[16] On the other hand, its gallate esters, for example, theaflavin mono/digallates (**9**, **10**, and **11** in Figure 1) underwent losses of 152 and 170 Da, indicating the loss of galloyl moieties. The whole series underwent oxygen insertions within the sites of the fragment ions obtained or within the available sites of the galloyl neutral losses having the same fragmentation mechanism. Hence, it was suggested that it is reasonable to expect that a similar fragmentation mechanism will apply to all members of the homologous series investigated. There is ample evidence in the literature for many classes of compounds, where minor structural changes do not alter the basic fragmentation pathways.[22,23] Therefore, the hydroxylated derivative will fragment by the same mechanism as the parent compound. It was anticipated that generally retention time on reversed phase packings would decrease relative to the parent compound as hydroxylation increased unless internal hydrogen bonding significantly increased the hydrophobicity.[16]

Theanaphthaquinone and Its Homologous Series. Theanaphthaquinone (**8**) showed an MS spectrum at m/z 533 characterized by its MS^2 fragment ions: at m/z 514 as a base peak revealing the loss of H_2O; at m/z 464 having a neutral loss of 69 Da, which is rationalized by the loss of C_3HO_2 from the quinone part of this compound; a fragment ion of a very weak intensity at m/z 396 showing a neutral loss of 137 Da indicating a RDA fragmentation occurring on one of the catechin moieties of this molecule. The EIC of theanaphthaquinone and its fragment spectra are shown in Figure 1 in the Supporting Information. Theanaphthaquinones have different sites available to hydroxylation, and to evaluate the assignment of these peaks as hydroxy-theanaphthaquinone, EICs were prepared to their corresponding predicted masses and the data are exhibited in Table 1. Furthermore, by using constant neutral loss analysis of the tandem mass, the presence of particular fragment ions and their neutral losses corresponding to their chromatographic peaks could be located and tentative structures assigned.

EICs were created for the predicted polyhydroxylated compounds of theanaphthaquinone (**8** + O_x). These showed the presence of m/z values corresponding to the components with two oxygen insertions at m/z 565.1 ($C_{28}H_{21}O_{13}$), three oxygen insertions at m/z 581.2 ($C_{28}H_{21}O_{14}$), four oxygen insertions at m/z 597 ($C_{28}H_{21}O_{15}$), and five oxygen insertions at m/z 613.1 ($C_{28}H_{21}O_{16}$). However, these were of low intensity when compared to other signals corresponding to members of homologous series of TFs and TCs previously reported.[17] The high-resolution masses of all TNQ derivatives were in line with data from previous FT-ICR-MS data and confirmed the elemental composition of these ions.[9]

Only a single chromatographic peak was detected for m/z 565.4 corresponding to **8** + O_2 for the predicted dihydroxylated derivative at a retention time of 50.1 min. Tandem mass experiment of this compound gave MS^2 fragment ions at m/z 544.9, revealing a loss of H_2O molecule, and a characteristic fragment ion at m/z 494.5, revealing a loss of C_3HO_2. The same fragmentation was previously observed in the parent ion, thus showing that this compound belongs to the homologous series of theanaphthaquinone with two oxygen insertions. This peak was tentatively assigned as a dihydroxylated derivateive of TNQ (**8** + O_2).

Four chromatographic peaks were detected in the EIC at the m/z 581.2 (**8** + O_3) at different retention times of 53.5, 43, 34.7, and 30.9 min, as expected for the trihydroxylated theanaphthaquinone. All exhibited an MS^2 fragment ion at m/z 512.5 as a base peak revealing a loss of 69 Da, thus

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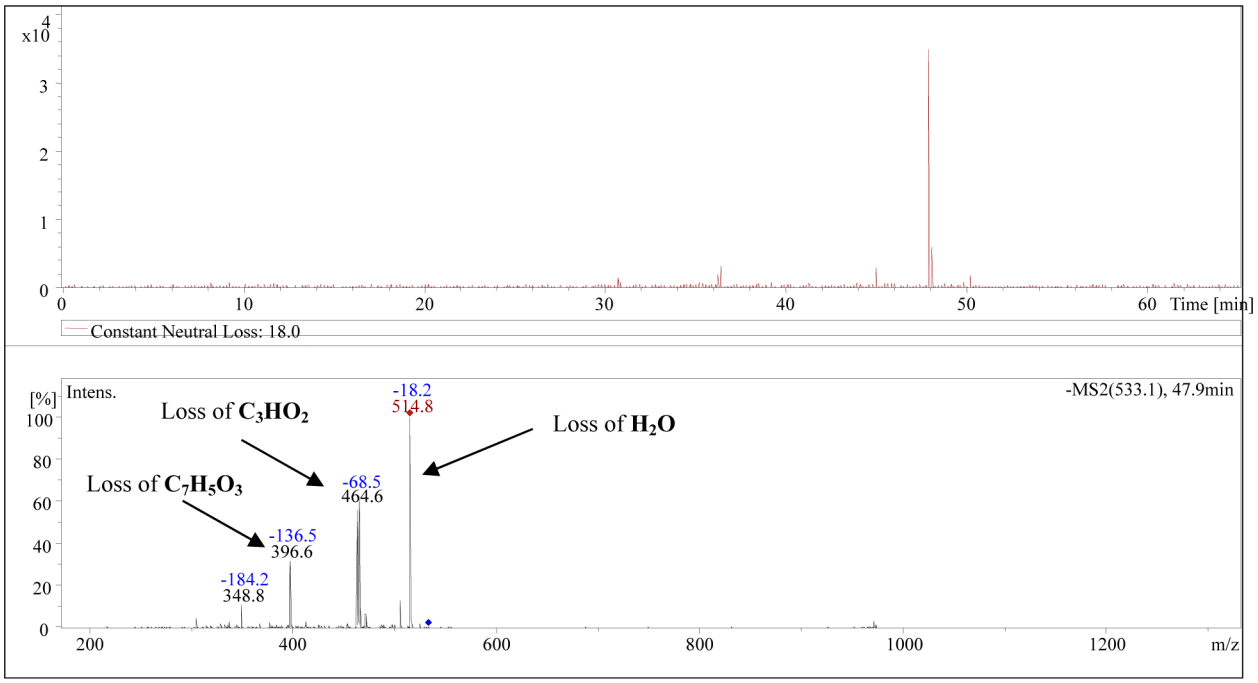

Figure 2. Neutral losses of H_2O and C_3HO_2 for theanaphthaquinone (**8**) at *m/z* 533.

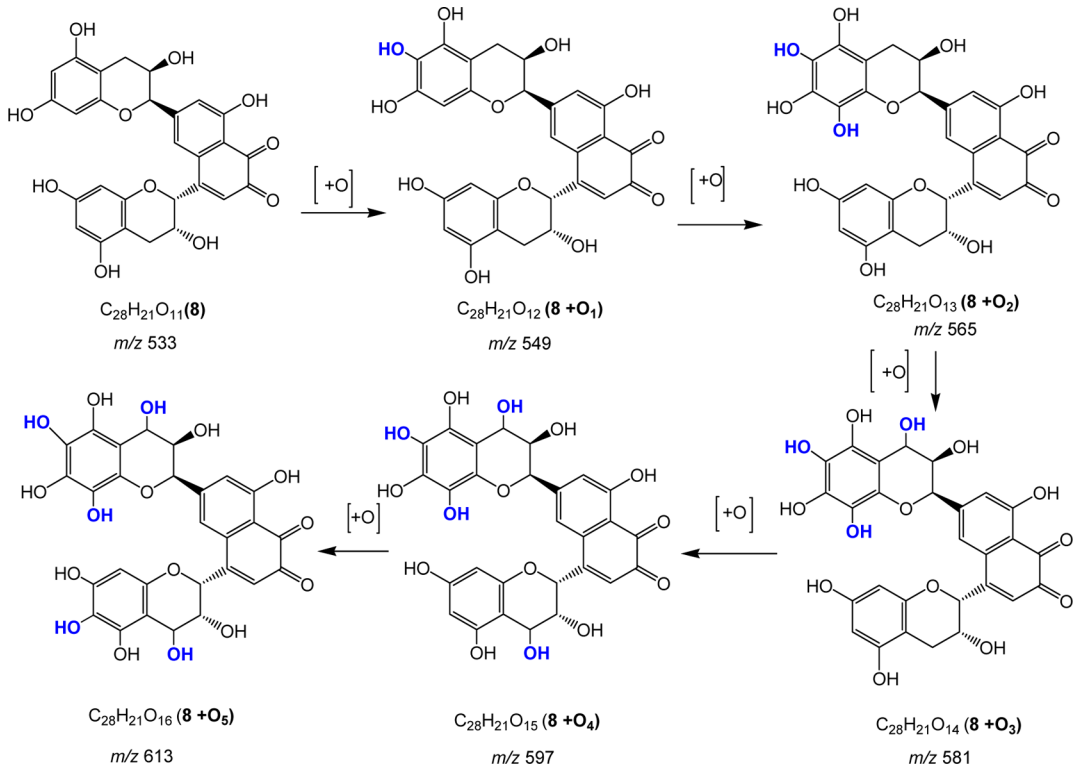

Figure 3. Homologous series of theanaphthaquinone (**8**) with successive oxygen insertion (regioisomers selected randomly).

corresponding to the loss of C_3HO_2 and revealing three oxygen insertions within the compound. Minor fragment ions with different intensities at *m/z* 444.5 were also observed revealing RDA fragmentation as previously identified in the parent ion. These four peaks were tentatively assigned as tetrahydroxylated derivatives of TNQ (**8** + O_4).

One chromatographic peak was detected for the *m/z* 597 at a retention time of 48.4 min corresponding to **8** + O_4. Tandem mass experiments showed a transition of this precursor ion to a fragment MS^2 ion at *m/z* 528.5 as a base peak characterized by a neutral loss of 69 Da of C_3HO_2 and revealing four oxygen insertions within this compound.

Two chromatographic peaks were detected for the *m/z* 613 at retention times of 39 and 28.1 min as expected for the pentahydroxylated theanaphthaquinone. Tandem mass fragmentation experiments showed a main fragment ion MS^2 at *m/*

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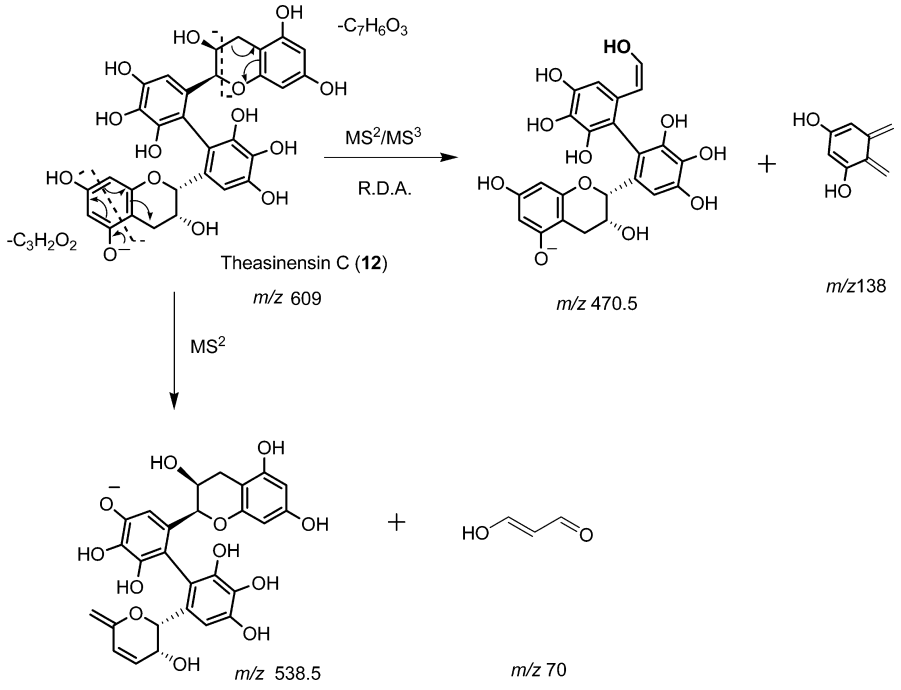

Figure 4. Fragmentation mechanism of theasinensin C (**12**) at m/z 609 undergoing loss of $C_3H_2O_2$ and retro-Diels–Alder fragmentation. (The sites of deprotonation in $[M - H]^-$ have been selected randomly.)

Table 2. Homologous Series for Theasinensin C (12) Confirming the Presence of the Hydroxylated Derivatives with Their Selected MS^2 and MS^3 Fragment Ions and Neutral Losses Observed[a]

	molecular formula	LC-MS $[M - H]m/z$	RT[b] (min)	fragment ions LC-MS^2 m/z	neutral loss $\delta M(S^1 - S^2)$[c] (Da)	fragment ions LC-MS^3 m/z
12	$C_{30}H_{25}O_{14}$	609.0	34.6	538.6 (67.8%), 456.9 (22.56%), 438.9 (41.1%)	70.4, 152.1, 170.1	
		609.0	34.1	**538.5 (100%)**, 470.5 (15.56%)	70., 138.5	470.5 (100%)
12 + O_2	$C_{30}H_{25}O_{16}$	641.0	33.2	604.8 (100%), **570.5 (92%)**	36.2, 70	504.5 (100%), 436.5 (26.2%)
				504.5 (21.5%)	137	
12 + O_4	$C_{30}H_{25}O_{18}$	673.1	20.1	654.1 (7%), **602.5 (100%)**	19, 70	544.5 (100%)
		673.1	40.1	**604.5 (100%)**, 535.5 (20.4%)	69, 136.6	544.5 (100%), 492.8 (15.8%)
		673	40.9	**655 (100%)**, 626.6, 604.5 (22.3%)	18,46, 69	502.9 (100%)
12 + O_6	$C_{30}H_{25}O_{20}$	705.6	47.8	**636.4 (100%)**	69.2	578.4 (100%), 520.5 (28.3%)

[a]MS^2 fragment ions highlighted in bold are subjected for further fragmentation for MS^3 fragment ions. [b]RT, retention time (min). [c]$\delta M(S1 - S2)$: mass loss (Da).

z 544.5 having a neutral loss of 69 Da of C_3HO_2, thus indicating all of the oxygens are inserted in the fragment ion, which corresponds to **8** + O_5.

The main neutral losses of all the chromatographic peaks identified revealing losses of H_2O, loss of C_3HO_2, and weak RDA fragmentation are shown in Figures 2 and 3, thus confirming the assignment made. Therefore, theanaphthaquinone undergoes nucleophilic addition of H_2O in the fermentation process for TR formation, where successive oxygen insertion takes place, giving its polyhydroxylated derivatives. These are exhibited in Figure 3, showing the hydroxylation process. Again, it should be noted that in the absence of authentic reference compounds, assignments of detailed structures are tentative and information on the regiochemistry of oxygen insertion is speculative with structures shown corresponding to positions of random oxygen insertion. Therefore, a new homologous series of theanaphthaquinone was observed within the oxidative cascade hypothesis extending the members of the thearubigin family.[16,17]

Theasinensin C and Its Homologous Series. Another systematic search for homologous series was carried out on theasinensin C ($C_{30}H_{25}O_{14}$) (**12**) shown in Figure 1 as a parent compound. Signals corresponding to m/z values of polyhydroxylated theasinensins were previously identified in FT-ICR-MS and MALDI-MS data of TR extracts.[16,24] EICs were also generated for the mass of parent ions expected for this homologous series. Theasinensin C (**12**) is characterized by it mass spectrum with $[M - H]$ ion at m/z 609, revealing the presence of two isomers at different retention times of 34.6 and 48.1 min, with the major chromatographic peak of this compound detected at 34.6 min as shown in Figure 2 in the Supporting Information. Performing tandem MS experiments with the precursor ion at m/z 609 gave a MS^2 fragment spectra characterized by a base peak at m/z 538.6 exhibiting a neutral loss of 70 Da, thus indicating a loss of $C_3H_2O_2$. Further MS^3

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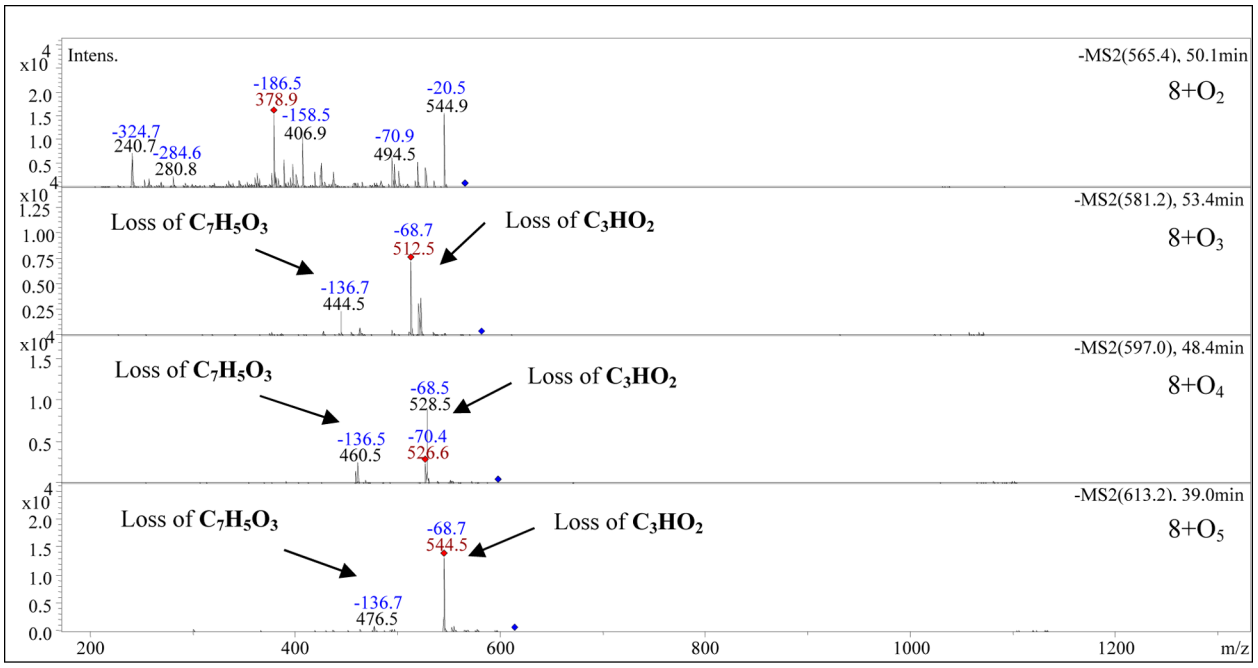

Figure 5. Constant neutral losses for the hydroxylated components of theanaphthaquinones (**8** + O_x): *m/z* 565.4, 581.2, 597.2, and 613.2 exhibiting losses of H_2O, C_3HO_2, and retro-Diels–Alder fragmentation.

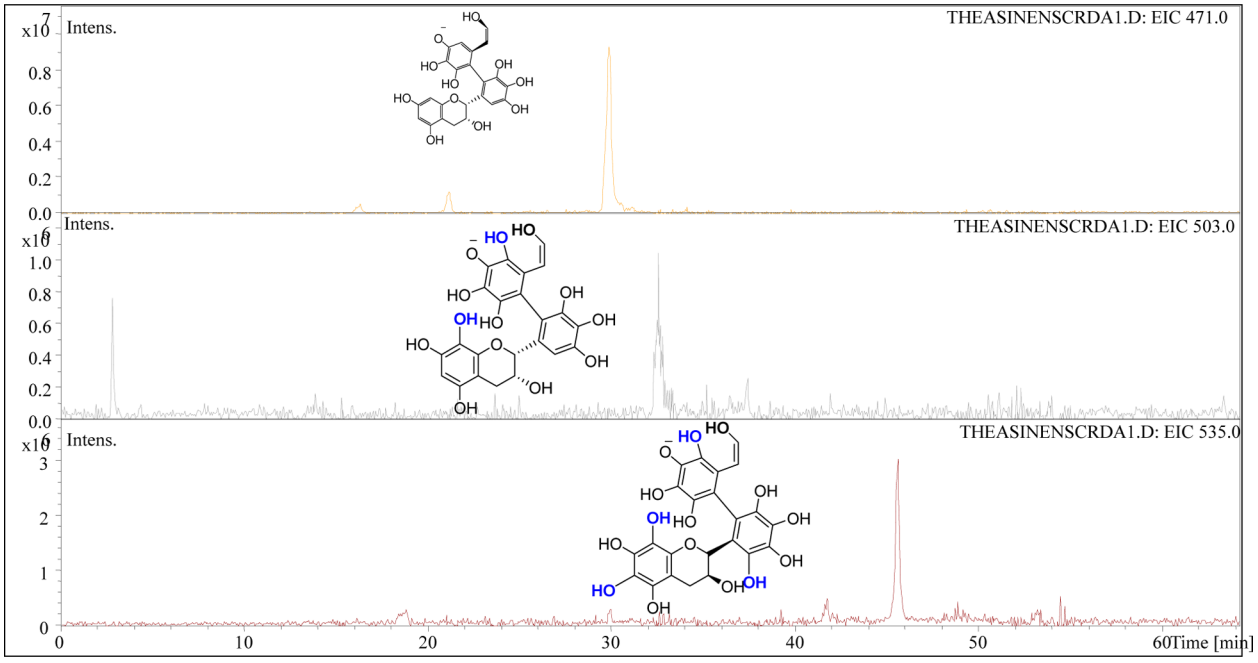

Figure 6. Extracted ion chromatograms EICs showing the retro-Diels–Alder (RDA) fragment ions in the homologous series of theasinensin C (**12**); (a, top) EIC at *m/z* 471; (b, middle) EIC at *m/z* 503; (c, bottom) EIC at *m/z* 535. (The sites of deprotonation in $[M - H]^-$ and regioisomers are selected randomly.)

tandem mass experiments with a precursor ion at *m/z* 538.6 gave the characteristic MS^3 fragment ion at *m/z* 470.5 corresponding to a loss of 138 amu, and this indicates that the molecule underwent first a loss of $C_3H_2O_2$, with fragmentation on one of the two benzopyran A-ring moieties followed by a RDA fragmentation with the loss of an enone fragment. The fragmentation mechanism is shown in Figure 4.

It is worth noting that while theasinensins have a biphenyl connection on the catechol B-rings, proanthocyanidins (another dimeric catechin structure) are connected from the resorcinol A-ring to the C-ring as indicated in Figure 5 in the Supporting Information. Both classes of compounds are constitutional isomers of one another with proanthocyanidins as well reported previously in tea chemistry. Both can be identified and differentiated by their different MS^2 fragment ions obtained in LC tandem MS. Proanthocyanidins show different MS^2 fragment spectra characterized by retro Diels–Alder fragmentation R.D.A. with a neutral loss of 168 Da giving *m/z* 441, Quinide Methide fission Q.M. giving *m/z* 305, and heterolytic ring fission H.R.F. giving an *m/z* 483 with a neutral

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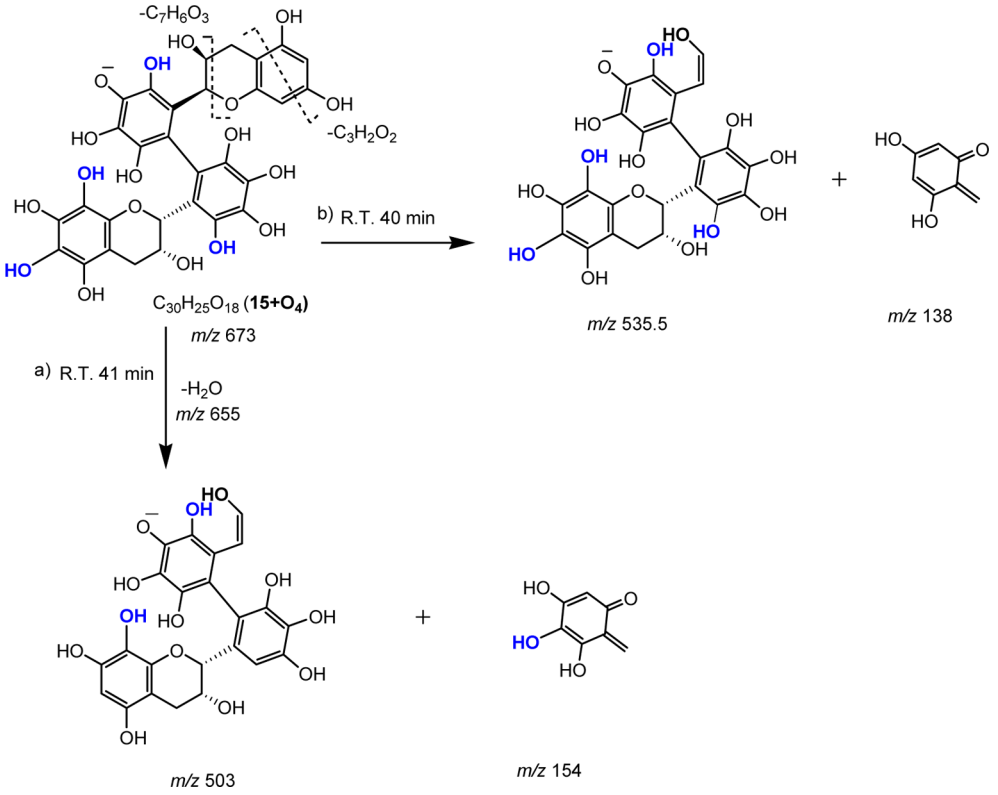

Figure 7. Mechanism of retro-Diels–Alder fragmentations (RDA) of a selected member of theasinensin C of the tetrahydroxylated derivative at *m/z* 673 showing the oxygen insertion process occurring. (The sites of deprotonation in $[M - H]^-$ and regioisomers are selected randomly.)

loss of 126 Da. Further details for differentiating between these two isomeric structures are shown in the fragmentation mechanism of the proanthocyanidin isomeric structure in Figure 6 in the Supporting Information.

To evaluate the assignment of the hydroxylated components within the homologous series of theasinensin C (**12**), EICs were prepared for their corresponding expected *m/z* values. Whereas theasinensin C (**12**) $C_{30}H_{25}O_{14}$ was detected at *m/z* 609, the EICs of its hydroxylated components were detected at *m/z* 641 for $C_{30}H_{25}O_{16}$, at *m/z* 673.1 for $C_{30}H_{25}O_{18}$, and at *m/z* 705.6 for $C_{30}H_{25}O_{20}$. The identity of the molecular formulas was previously established by FT-ICR-MS.[9] A detailed study for the hydroxylated derivatives of this compound and their MS^2 and MS^3 fragment ions is exhibited in Table 2.

One chromatographic peak was identified for *m/z* 641 $C_{30}H_{25}O_{16}$, corresponding to theasinensin C with two oxygen insertions (**15** + O_2), eluting at a retention time of 33.2 min. This compound gave a fragment ion at *m/z* 604.8 as a major peak exhibiting a neutral loss of 36 amu, corresponding to a loss of two H_2O molecules from this ion, and another characteristic MS^2 ion at *m/z* 570.5 revealing the loss of $C_3H_2O_2$. Further fragmentation gave MS^3 as base peak at *m/z* 504.5 revealing the further RDA fragmentation occurring in this compound. Hence, the fragmentation pattern observed match fragment ions expected for a dihydroxylated theasinensin derivative.

Three isomers in the EIC with *m/z* 673 were detected at different retention times of 20.1, 40.1, and 40.9 min, corresponding to the predicted tetrahydroxylated theasinensin **12** + O_4 ($C_{30}H_{25}O_{18}$). One of them eluting at 20.1 min showed a transition from *m/z* 673.1 to a fragment ion at *m/z* 602.5 as a base peak, indicating the loss of $C_3H_2O_2$ and the oxygen insertions within the dimeric ring. The second isomer of the *m/z* 673 eluting at 40.1 min showed a transition from *m/z* 673 to 604.5 and 535.5, exhibiting a major loss of $C_3H_2O_2$ on the benzopyran ring and a minor loss of 136.6 amu due to RDA fragmentation occurring, whereas all of the hydroxyl groups are inserted within the fragment ion of the theasinensin C ring. The third isomer of the *m/z* 673 was eluting at 40.9 min showing a transition from *m/z* 673 to 655 exhibiting a neutral loss of 18 amu, which is the loss of water, and indicating three hydroxyl insertions within this compound. A further MS^3 fragmentation for this compound gave a transition of the MS^2 fragment ion at *m/z* 655 to MS^3 at *m/z* 503, which indicates that two hydroxyl groups are inserted within the theasinensin fragment ion and one hydroxyl insertion was occurring within the eliminated benzopyran moiety, whereas this molecule was undergoing a loss of water at the same time. This is briefly illustrated in Figure 7. Hence the fragmentation pattern observed matches the fragment ions expected for the tetrahydroxylated theasinensin derivative. In a part of fragmentation pattern of these compounds a loss of water was observed, which has not been observed in the parent compound. An additional set of EICs were created in the all MS^n mode searching for fragment ions that would be characteristic for hydroxylated theasinensins. Representative data are shown in Figure 6. Here fragment ions originating from RDA fragmentations at *m/z* 471, 503, and 535 were observed corresponding to bisflavanol-like compounds with two or four oxygens inserted, respectively. These observed fragment ions point toward the presence of hydroxylated theasinensins. It should as well be noted that the fragment spectra obtained showing frequently loss of two water molecules from the precursor ion might as well correspond to hydroxylated theasinensin derivatives, in which, due to a

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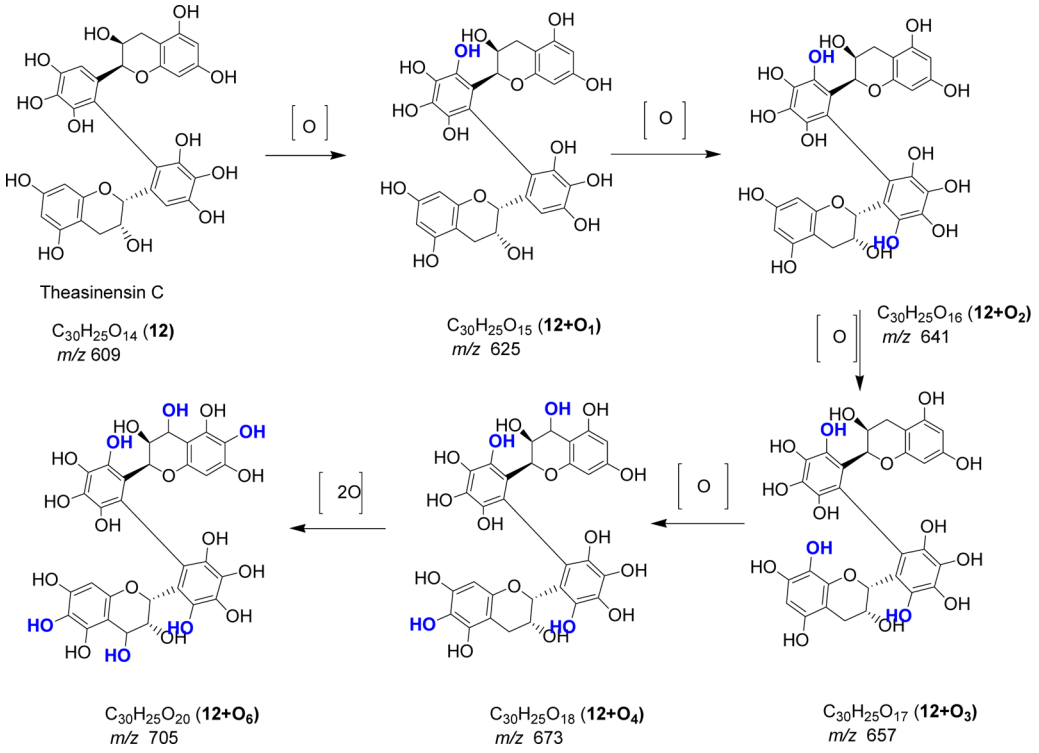

Figure 8. Homologous series of theasinensin C (**12**) with successive oxygen insertion. (Regioisomers are selected randomly.)

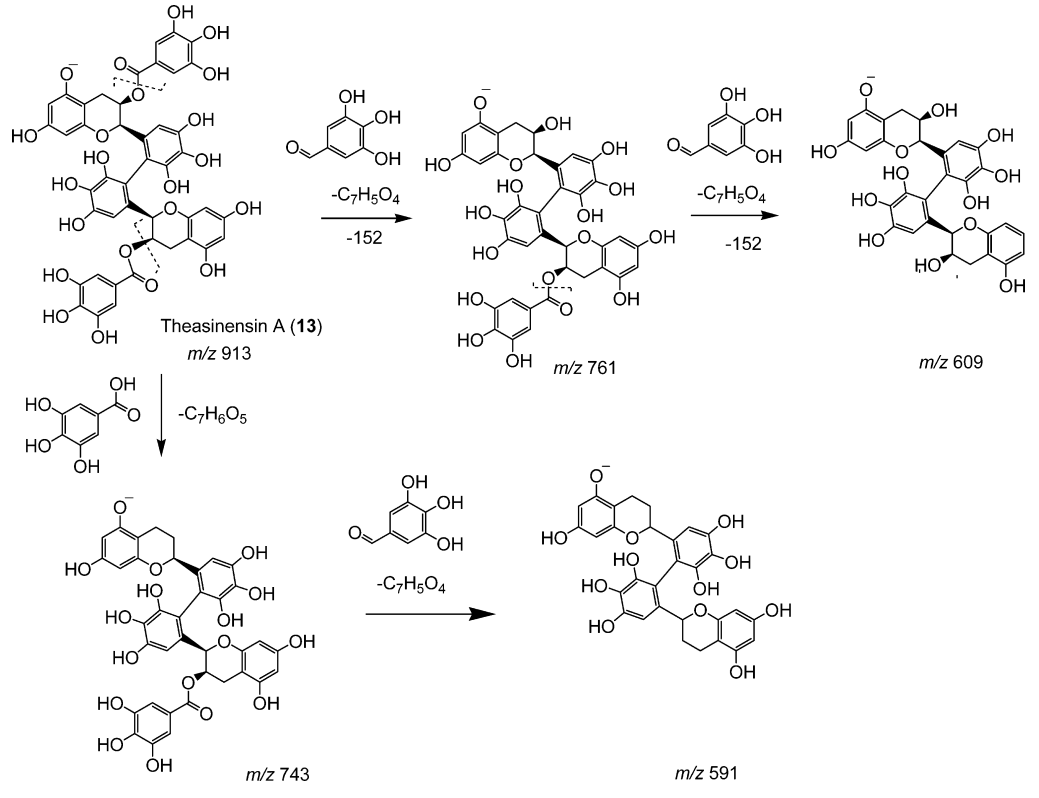

Figure 9. Mechanism of fragmentation for theasinensin A (**12**) at m/z 913.1 exhibiting its characteristic fragment ions. (The sites of deprotonation in $[M - H]^-$ and regioisomers are selected randomly.)

change of the aromatic substitution pattern, the fragmentation pathway is altered.

Only one isomer of $C_{30}H_{25}O_{20}$ corresponding to **15** + O_6 was observed at m/z 705.6, giving a transition to m/z 636.4 as a major peak intensity, which reveals a loss of $C_3H_2O_2$ from the A-ring and indicating all of the hydroxyl groups are inserted within the theasinensin fragment.

All of the above-mentioned results are exhibited in Table 2, confirming the presence of a new homologous series for theasinensin C present in thearubigin fraction. Tetntative

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Table 3. New Class of Compounds for Theasinensin A (13) Detected with Their Selected Tandem Mass Spectral Data and Their Neutral Losses, Showing Different Fragmentation Mechanisms Compared to the Parent Ion

	molecular formula	LC-MS [M − H] m/z	RT[a] (min)	fragment ions LC-MS^2 m/z	neutral loss δM($S^1 - S^2$)[b] (Da)
13	$C_{44}H_{33}O_{22}$	913.2	22.1	761.2 (28%), 743.2 (40.59%), 609.1 (5.44%), 591.2 (100%)	152, 170, 304, 322
			16.3	761.2 (34.01%), 743.2 (23%), 609 (14.92%), 591.2 (100%)	152, 170, 304, 322
			15.7	761.2 (39.67%), 743.2 (33.17),, 609.2 (15.1%), 591.2 (100%)	152, 170, 304, 322
			1.7	761.1 (32.29%), 743.2 (31.27%), 609.2 (13.98%), 591.2 (100%)	152, 170, 304, 322
13 + O_1	$C_{44}H_{33}O_{23}$	929.0	32.6	913.3 (76.06%), 870.7 (100%)	16, 58.3
13 + O_2	$C_{44}H_{33}O_{24}$	945.0	52.3	926.6 (11.78%), 909.1 (100%), 657.2 (8%) (609 + 3O)	18, 36, 287.8
		945.3	47.8	909.2 (26%), 886.6 (100%)	36, 58.4
13 + O_3	$C_{44}H_{33}O_{25}$	960.9	64	916.9 (100%), 657 (12.4%) (609 + 3O)	44, 304
13 + O_4	$C_{44}H_{33}O_{26}$	977.1	51.2	959.4 (19.1%), 920.6 (100%)	17.5, 56
		977.3	32.8	918.5 (57.8%), 863.6 (22.68%)	58.1, 113
13 + O_5	$C_{44}H_{33}O_{27}$	993.3	49	961.2 (19.2%), 934.6 (100%)	32, 58.4

[a]RT, retention time (min). [b]δM(S1 − S2), mass loss (Da).

structures of the homologous series of theasinensin C are shown in Figure 8.

Theasinensin A and Its Homologous Series. Theasinensin A (**13**) as indicated in Figure 1 is characterized by its mass spectrum with a pseudomolecular ion at m/z 913.2 [M − H] in the negative ion mode, exhibiting different characteristic fragment ions. In the EIC of m/z 913 as shown in Figure 8 in the Supporting Information, three signals are observed at different retention times of 1.7, 16.3, and 22.5 min. The first fragment ion was observed at m/z 761.2 with a neutral loss of 152 amu, and a second fragment ion was also observed at m/z 743.2 with a neutral loss of 170 amu, both revealing the loss of one of the galloyl moieties of this compound. A third fragment ion was observed at m/z 609.1, exhibiting a loss of 304 amu, which can be rationalized due to a loss of two galloyl moieties from both parts of theasinensin A. A base peak fragment ion was exhibited in the MS^2 spectra at m/z 591.2, indicating the loss of one galloyl group from one part of the molecule and another gallate ester from the second part. The fragmentation of this compound and its mechanism are shown briefly in Figure 9.

To evaluate the assignment of the hydroxylated components within the homologous series of theasinensin A (**13**), EICs were prepared for their corresponding expected m/z values. Whereas theasinensin A (**13**) was detected at m/z 913.2 ($C_{44}H_{33}O_{22}$), EICs for its predicted hydroxylated components were prepared at m/z 929 ($C_{44}H_{33}O_{23}$), at m/z 945 ($C_{44}H_{33}O_{24}$), at m/z 961 ($C_{44}H_{33}O_{25}$), at m/z 977.1 ($C_{44}H_{33}O_{26}$), and at m/z 993.3 ($C_{44}H_{33}O_{27}$). These results are shown in Table 3. Targeted tandem MS experiments of the hypothetical hydroxylated components of theasinensin A (**13**) were carried out for evaluating the presence of oxygen insertions within these compounds, to confirm whether a homologous series of theasinensin A is present.

As shown in Table 3, the EIC at m/z 929 showed a single peak at a retention time of 32.6 min corresponding to a theasinensin A with one oxygen inserted (**13** + O_1). The precursor ion at m/z 929.1 displayed a MS^2 fragment ion at m/z 913.3 as a base peak having a neutral loss of only 16 amu. However, such MS^2 fragmentation did not show a similar fragmentation as the parent ion of theasinensin A, undergoing the neutral losses of the galloyl moieties, which is its characteristic fragmentation mechanism. Hence, this could not be in agreement with the hypothesis of the homologous series as previously described by which the whole series undergoes similar if not identical fragmentation as described for the parent ion. Neutral loss of 16 amu is characteristic for either peroxide functionalities or epoxide functionalities, so that the compound observed was tentatively assigned to a peroxo or epoxy derivative of a bisflavanol.

Two chromatographic peaks corresponding to the predicted dihydroxylated component of theasinensin A (**13** + O_2) were detected in the EIC at m/z 945.0. The first peak was observed at a retention time of 52.3 min, where its MS^2 spectrum exhibited fragment ions at m/z 926.6 showing a loss of H_2O, a base peak at m/z 909.1 with a neutral loss of 36 amu, thus indicating a further loss of H_2O molecule, and a characteristic fragment ion with a very weak intensity at m/z 657.2, revealing a loss of two galloyl moieties from this compound undergoing a loss of oxygen ($2C_7H_5O_4 - O$) and three oxygen insertions within the fragment ion, which is in agreement as the fragmentation pathway of the parent ion theasinensin A. A second isomer of m/z 945 was detected at a retention time of 47.8 min; the MS^2 spectrum of this compound gave a fragment ion with a medium intensity at m/z 909.2 revealing a loss of two molecules of H_2O.

Only one chromatographic peak in the EIC at $m/$ 961 corresponding to three oxygen insertions (**13** + O_3) was observed at 64.0 min. Its MS^2 spectrum showed a characteristic base peak at m/z 916.9 suffering a neutral loss of 44 Da (loss of CO_2), and further fragment ions were observed, one of which was detected coeluting with a low intensity at m/z 657 exhibiting a neutral loss of 304 Da, which indicates the loss of the two galloyl groups from this compound as in the parent ion and three oxygen insertions within the fragment ion. The latter fragment ion is in agreement with a trihydroxylated theasinensin structure.

Two chromatographic signals in the EIC at m/z 977.1 for the predicted tetrahydroxylated derivatives of theasinensin A (**13** + O_4) were detected. The first one was observed at a retention time of 51.2 min, exhibiting MS^2 fragment spectra characterized by a weak fragment ion at m/z 959.4 having a neutral loss of 17.5, which corresponds to a loss of one hydroxyl group, and another characteristic MS^2 ion with high intense peak was identified at m/z 920.6 having a neutral loss of 56 Da ($-C_3H_4O$) characteristic for flavanol C-ring cleavage. The second isomer at m/z 977.3 was observed eluting at 32.8 min, and the latter showed no characteristic fragmentation of

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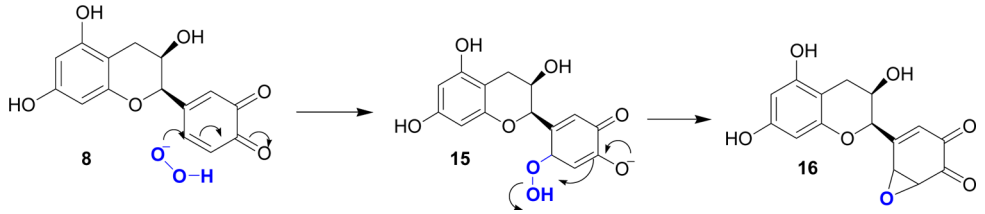

Figure 10. Proposed mechanism undergoing oxygen insertion by nucleophilic addition of H_2O_2.

theasinensin A. Thus, these two mentioned isomeric compounds could not be considered as a part of the homologous series of theasinensin A, so no tetrahydroxylated components of this series are present.

A single chromatographic peak was observed in the EIC at m/z 993.3, a value predicted for a pentahydroxylated derivative of theasinensin A (**13** + O_5). The MS^2 spectrum of this component exhibited a characteristic fragment ion at m/z 961.2 showing a neutral loss of 32 amu, which indicates the loss of two oxygens, whereas only three oxygens were inserted within the fragment ions of this compound. As in the previously identified compounds, this new component did not undergo the same fragmentation as the parent compound, theasinensin A, thus indicating that this compound does not belong to the homologous series suggested.

Accordingly, this observation can be rationalized by suggesting that a new series of oxidation products in thearubigin formation may exist. These new compounds undergo mainly oxygen losses (−32 Da, $-O_2$) during MS^2 is a characteristic for peroxides or epoxides. It is proposed that these compounds can be formed in the presence of H_2O_2 in black tea thearubigin fractions, with hydrogen peroxide acting as a nucleophile adding to *o*-quinone moieties. It should be noted that due to the α-effect hydrogen peroxide is around 1000 times more nucleophilic if compared to water, the conventional nucleophile involved in thearubigin chemistry. The existence of H_2O_2 in black tea has been previously discussed and confirmed by Sinkar et al. in an attempt to investigate the biochemistry and chemistry of thearubigins using polyphenol oxidase and peroxidase enzymes.[20] In his work, Sinkar demonstrated that TPPO, while oxidizing catechins, generates H_2O_2 in concentrations of 290−390 μM, which may play a role in the oxidation process in the thearubigin formation. In this part of research, we were able to identify a further oxygenation process taking place but here by nucleophilic addition of H_2O_2 as the second suggested mechanism in the oxidation process for the formation of new peroxide intermediate (**15**) or epoxide structures (**16**) by nucleophilic attack of the enolate moiety in peroxo intermediate (**15**). The new suggested mechanism is shown in Figure 10.

Alternative Graphical Representation in Modified Kendrick Plot of Homologous Series. In our study new thearubigin components were clearly characterized by employing tandem LC-MS^n experiments. The data generated in the homologous series of theanaphthoquinone and theasinensin C were consistent with the hypothesis previously formulated, in which polyhydroxylated dimers of catechins are formed by nucleophilic addition of water to the quinone oxidized counterparts of catechins. On the other hand, new compounds containing oxygen insertions in the series of theasinensin A were identified, where the latter were rationalized according to the nucleophilic addition of hydrogen peroxide, thus forming thearubigin components of a different structural class. Data on the homologous series of theasinensin A were inconclusive, with MS data not allowing tentative structure suggestions. In summary, this study has complemented previous knowledge on thearubigin chemistry and added evidence for the presence of homologous series of formal oxygen insertion for the two previously not studied dimers of catechins, theasinensins and theanaphthaquinone. Most TR components identified so far must be considered as belonging to the class of flavonoids originating from flavonoid precursors all possessing at least one characteristic C6−C3−C6 moisty.

In previous work we have used conventional Kendrick diagrams using Kendrick masses plotted against Kendrick mass deficits as visual display tools.[9,16] However, this type of representation seems inadequate for black tea chemistry or in general any other processed food. Therefore, we introduce at this stage a modified graphical representation of homologous series reminiscent of a mass deficit diagram in Figure 11. In the

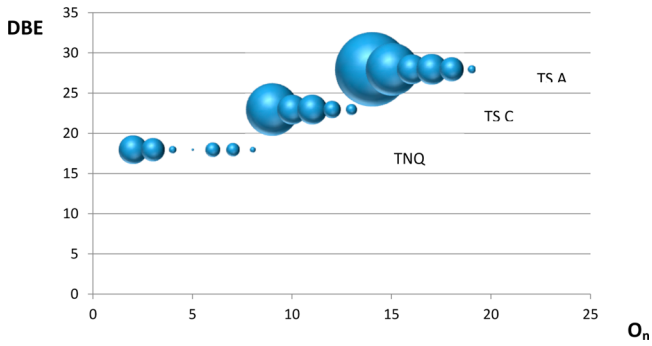

Figure 11. Modified Kendrick diagram with numbers of oxygens O_n plotted against double-bond equivalents (DBE) displaying homologous series of theanaphthoquinone (TNQ) and theasinensins A and C (TS A and TS C) with bubble size corresponding to absolute intensities from direct infusion mass spectral data in the negative ion mode (see ref 16).

case of TR components homologous series differ in number of oxygen atoms, which are plotted on the *x*-axis. On the *y*-axis a compound class-specific parameter needs to be chosen. We suggest using double-bond equivalents (DBEs) as such a parameter. In certain cases, for example, if comparing TFs with TSs, the number of DBE (18 in this case) is identical, so for such situations we suggest using as a compound class-specific parameter, the number of carbon atoms C_n divided by DBEs. As an additional third parameter in Figure 11 the intensity of the mass spectral peak corresponding to the member of the homologous series is shown in a bubble plot with intensity corresponding to the diameter of the bubble.

Such a modified Kendrick diagram with numbers of oxygens O_n plotted against DBE displaying homologous series of theanaphthoquinone (TNQ) and theasinensins A and C (TS A

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and TS C) with bubble size corresponding to absolute intensities on parallel lines to the *x*-axis from direct infusion mass spectral data in the negative ion mode[16] is introduced here.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Figures 1−5. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ESI/LC-MS^n, electrospray ionization/high-performance liquid chromatography−tandem mass spectrometry; EIC, extracted ion chromatograms; TRs, thearubigins

■ REFERENCES

(1) Poulter S. Daily Mail online, dailymail, UK, June 27, 2008.

(2) FAO Web site with data from 2013:, http://www.fao.org/news.

(3) Drynan, J. W.; Clifford, M. N.; Obuchowicz, J.; Kuhnert, N. The chemistry of low molecular weight black tea polyphenols. *Nat. Prod. Rep.* **2010**, *27* (3), 417−462.

(4) Kuhnert, N.; Dairpoosh, F.; Yassin, G.; Golon, A.; Jaiswal, R. What is under the hump? Mass spectrometry based analysis of complex mixtures in processed food − lessons from the characterisation of black tea thearubigins, coffee melanoidines and caramel. *Food Funct.* **2013**, *4*, 1130−1147.

(5) Roberts, E. A. H.; Wood, D. J. Separation of tea polyphenols on paper chromatograms. *Biochem. J.* **1953**, *53*, 332−336.

(6) Kallithraka, S.; Bakker, J.; Clifford, M. N. Evaluation of bitterness and astringency of (+)-catechin and (−)-epicatechin in red wine and in model solution. *J. Sens. Stud.* **1997**, *12*, 25−37.

(7) Bruschi, L.; Copeland, E. L.; Clifford, M. N. Unexpected hyperchromic interactions during the chromatography of theafulvins and simple flavonoids. *Food Chem.* **1999**, *67*, 143−146.

(8) Gardener, E. J.; Ruxton, C. H. S.; Leeds, A. R. Black tea − helpful or harmful? A review of the evidence. *Eur. J. Clin. Nutr.* **2006**, 1−16.

(9) Kuhnert, N. Unraveling the structure of the black tea thearubigins. *Arch. Biochem. Biophys.* **2010**, *501*, 37−51.

(10) Wu, Z. G.; Rodgers, R. P.; Marshall, A. G. Compositional determination of acidic species in Illinois no. 6 coal extracts by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. *Energy Fuels* **2004**, *18*, 1424−1428.

(11) Kim, S.; Kramer, R. W.; Hatcher, P. G. Graphical method for analysis of ultrahigh-resolution broadband mass spectra of natural organic matter, the van Krevelen diagram. *Anal. Chem.* **2003**, *75*, 5336−5344.

(12) Kendrick, E.; Mass, A. Scale based on CH_2 = 14.0000 for high resolution mass spectrometry of organic compounds. *Anal. Chem.* **1963**, *35*, 2146−2152.

(13) Hughey, C. A.; Rodgers, R. P.; Marshall, A. G. Resolution of 11000 compositionally distinct components in a single electrospray ionization Fourier transform ion cyclotron resonance mass spectrum of crude oil. *Anal. Chem.* **2002**, *74*, 4145−4149.

(14) Hughey, C. A.; Galasso, S. A.; Zumberge, J. E. Detailed compositional comparison of acidic NSO compounds in biodegraded reservoir and surface crude oils by negative ion electrospray Fourier transform ion cyclotron resonance mass spectrometry. *Fuel* **2007**, *86*, 758−768.

(15) Koch, B. P.; Dittmar, T.; Witt, M.; Kattner, G. Fundamentals of molecular formula assignment to ultrahigh resolution mass data of natural organic matter. *Anal. Chem.* **2007**, *79*, 1758−1763.

(16) Kuhnert, N.; Drynan, J. W.; Obuchowicz, J.; Clifford, M. N.; Witt, M. Mass spectrometric characterization of black tea thearubigins leading to an oxidative cascade hypothesis for thearubigin formation. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 3387−3404.

(17) Kuhnert, N.; Clifford, M. N.; Mueller, A. Oxidative cascade reactions yielding polyhydroxy-theaflavins and theacitrins in the formation of black tea thearubigins: evidence by tandem LC-MS. *Food Funct.* **2010**, *1*, 180−199.

(18) Yassin, G.; Koek, J.; Kuhnert, N. Identification of trimeric and tertameric flavan-3-ol derivatives in the SII black tea thearubigin fraction of black tea using ESI-tandem and MALDI-TOF mass spectrometry. *Food Res. Int.* **2014**, DOI: 10.1016/j.foodres.2014.04.010.

(19) Nakayama, T.; Ichiba, M.; Kuwabara, M.; Kajiya, K.; Kumazawa, S. Mechanisms and structural specificity of hydrogen peroxide formation during oxidation of catechins. *Food Sci. Technol. Res.* **2002**, *8*, 261−267.

(20) 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.

(21) Mulder, T. P. J.; van Platerink, C. J.; Schuyl, P. J. W.; van Amelsvoort, J. M. M. Analysis of theaflavins in biological fluids using liquid chromatography-electrospray mass spectrometry. *J. Chromatogr., B* **2001**, *760*, 271−279.

(22) Cuyckens, F.; Claeys, M. Mass spectrometry in the structural analysis of flavonoids. *J. Mass Spectrom.* **2004**, *39*, 1−15.

(23) Cren-Olive, C.; Deprez, S.; Lebrun, S.; Coddeville, B.; Rolando, C. Characterization of methylation site of monomethylflavan-3-ols by liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 2312−2319.

(24) Drynan, J. W.; Clifford, M. N.; Obuchowicz, J.; Kuhnert, N. MALDI-TOF mass spectrometry: avoidance of artifacts and analysis of caffeine-precipitated sill thearubigins from 15 commercial black teas. *J. Agric. Food Chem.* **2012**, *60*, 4514−4525.