MALDI-TOF Mass Spectrometry: Avoidance of Artifacts and Analysis of Caffeine-Precipitated SII Thearubigins from 15 Commercial Black Teas

J. Warren Drynan,[†,#] Michael N. Clifford,[‡] Jacek Obuchowicz,[§] and Nikolai Kuhnert*[,#]

[†]Division of Chemical Sciences and [‡]Division of Nutritional Sciences, Faculty of Health and Medical Sciences, University of Surrey, Guildford GU2 7XH, United Kingdom

[§]Unilever, Colworth Science Park, Sharnbrook MK44 1LQ, United Kingdom

[#]School of Engineering and Science, Chemistry, Jacobs University Bremen GmbH, Campusring 8, 28759 Bremen, Germany

Ⓢ *Supporting Information*

ABSTRACT: Thearubigins are the quantitatively major phenolic compounds in black tea, accounting for some 60−70% of the solids in a typical black tea infusion. MALDI-TOF mass spectra for caffeine-precipitated SII thearubigins (SII CTRs) from 15 different commercial teas support previous conclusions that SII CTRs are polyhydroxylated oligomers (rather than polymers) of catechins and catechin gallates in redox equilibrium with their quinone counterparts. Some 4500 peaks were revealed in a mass range from m/z 500 to 2100. Polyphenols are redox-susceptible and readily generate artifacts during MALDI-TOF analysis when the matrix is also redox-susceptible. Of the nine matrices evaluated, 3′,4′,5′-trihydroxyacetophenone (**F**) provided the best compromise between signal intensity and redox artifact formation.

KEYWORDS: *black tea, theaflavins, SII CTRs, polyphenols, mass spectrometry, matrices, interpretation strategies, MALDI artifacts*

■ INTRODUCTION

This paper is part of a series by our group directed to the structural elucidation of caffeine-precipitated SII thearubigins (SII CTRs). Aspects of the current paper have recently been published;[1] however, we now describe in detail our attempts to elucidate structural features of SII CTRs with the aid of MALDI-TOF mass spectrometry. In recent work we have detailed the isolation and characterization of SII CTRs from 15 different commercial teas by standard spectroscopic and analytical chemical methods. These methods consist of FT-IR spectroscopy, NMR spectroscopy, diffusion NMR spectroscopy, CD spectroscopy, UV−vis spectroscopy, ICP-OES, combustion analysis, and AFM. Furthermore, we have probed the chemical nature of SII CTRs by LC-MS[n] and direct infusion MS[n] and formulated a consistent hypothesis for their formation.[2] Our conclusions about characteristic SII CTR chemical structure and the mechanism of their formation can be summarized as follows:

(i) The SII CTR fraction of a commercial black tea comprises several thousand compounds, an order of magnitude higher than previously expected, with around 10000 molecular ions resolved in a single, direct infusion, ESI-FTICR-MS experiment. Around 1500 molecular formulas were assigned using these data. Compounds of molecular mass >1200 amu appeared in the spectrum as multiple-charged ions. For this reason, the total number of compounds remains unclear, and no molecular formula assignment could be carried out for these ions.

(ii) Petrolomics-style and novel data interpretation strategies were adapted and developed to allow the visualization and interpretation of enormously complex data.

(iii) Interpretation of the data allowed a structural hypothesis for the SII CTRs to be developed that suggests the formation of polyhydroxylated species, which are formed via oxidation, and nucleophilic addition of water and hydrogen peroxide to catechin dimers and oligomers. The catechin dimers and oligomers are formed via oxidative coupling between catechin monomers.

(iv) Furthermore, the observed molecular ions could be rationalized in terms of redox cascades wherein catechin oligomers are hydroxylated via *o*-quinones to yield polyhydroxylated species which, in turn, are in equilibrium with their quinone counterparts accounting for >90% of the molecular ions assigned. The existence of these redox cascades was supported by data from a range of other spectroscopic techniques.

(v) SII CTRs from different sources are remarkably similar with respect to all of their spectroscopic fingerprints.

(vi) SII CTRs display an unusual, Gaussian-shaped, hump in their reversed-phase HPLC chromatograms due to the large number of compounds present, the combined effect of noncovalent interactions such as hydrogen bonding and π−π stacking, and equilibration/re-equilibration during chromatography.

The importance of black tea in the world economy and the chemical basis for its manufacture, termed "tea fermentation", which comprises enzyme-mediated conversion of catechins **1−6**, via an initial two-electron oxidation, to various dimers such as
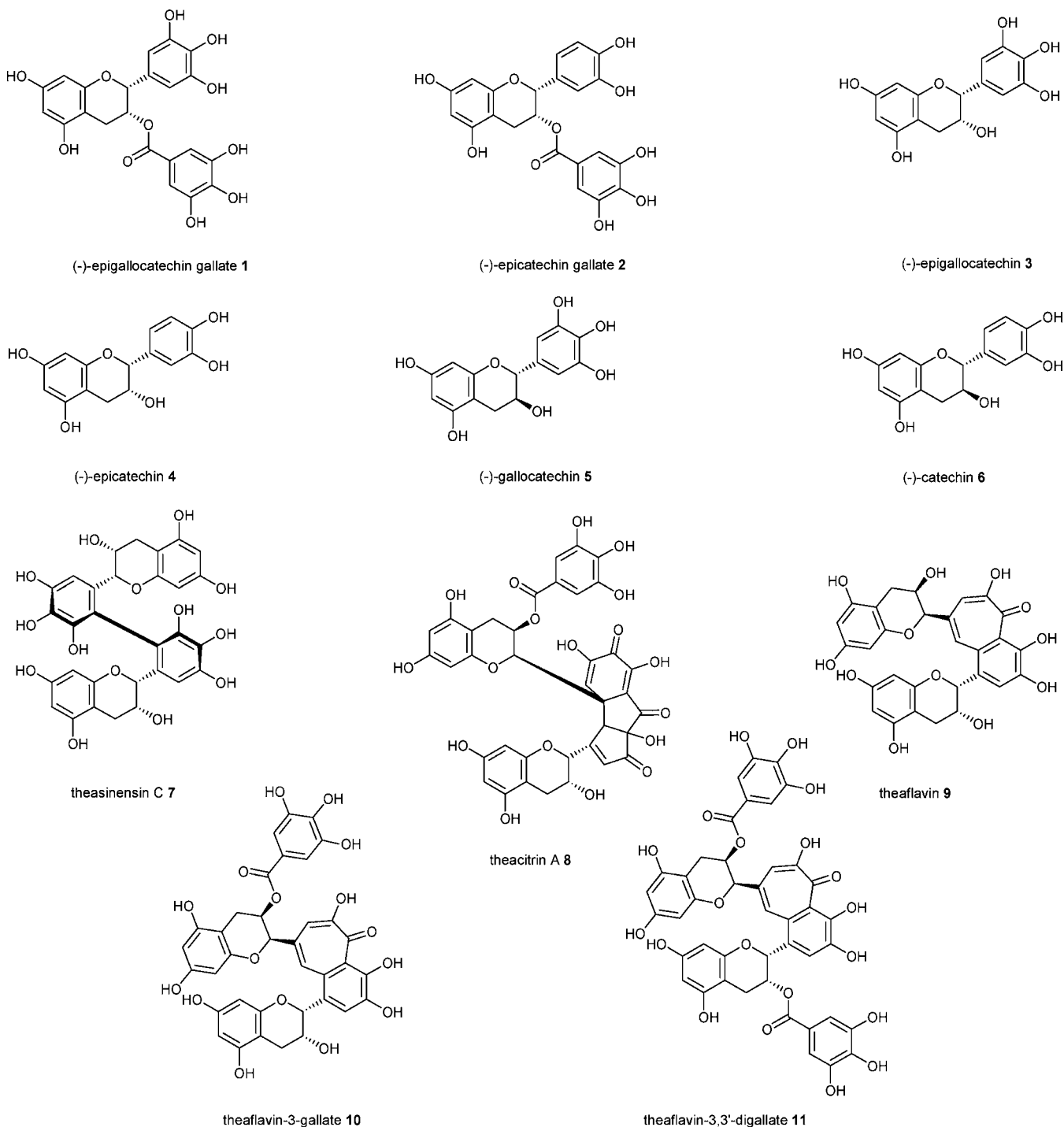
Chart 1. Structures of Catechins (1−6) and Catechin Dimers (7−11) Found in Black Tea

(-)-epigallocatechin gallate **1**

(-)-epicatechin gallate **2**

(-)-epigallocatechin **3**

(-)-epicatechin **4**

(-)-gallocatechin **5**

(-)-catechin **6**

theasinensin C **7**

theacitrin A **8**

theaflavin **9**

theaflavin-3-gallate **10**

theaflavin-3,3'-digallate **11**

7−11 (see Chart 1) has been discussed and reviewed in detail.[3,4] The discovery and history of the thearubigins has also been reviewed[5,6] and will not be repeated here. Suffice it to say that the thearubigins are chromatographically unresolvable, black tea quality-influencing polyphenols that account for 60−70% of the dry weight of a black tea infusion and their chemical structures remain incompletely known despite our recent advances.[6]

Whereas ESI-FTICR-MS offers unprecedented resolving power and mass accuracy, the technique suffers from the disadvantage that higher molecular mass ions escape detection because they appear as double- or multiple-charged ions in the mass range of monocharged ions. Accordingly, molecular formulas cannot be assigned. This disadvantage can be circumvented by using MALDI-TOF-MS which, despite having lower mass resolution, is known to produce, almost exclusively, monocharged ions during the ionization process, therefore allowing the upper reaches of the envelope of the molar mass distribution of thearubigins to be obtained. The selectivity of the MALDI process to produce, almost exclusively, mono-charged ions has been addressed by employing various ionization models including a matrix cluster ionization model, whereby ionization comprises initial formation of charged matrix−analyte clusters, which are then reduced via ion−ion

and/or neutral-ion chemical reactions to monocharged species,[7] and an energy transfer-induced disproportionation (ETID) ionization model.[8] Although the highest MALDI mass range is obtained in linear mode, reflector mode offers greater resolution and has been successfully employed to obtain single oligomer resolution of polystyrene up to a mass of 25000 amu when applied in conjunction with ion extraction delay.[9]

MALDI-TOF-MS has been applied to the analysis of complex oligomeric food polyphenols,[10−13] and the power of the technique to determine the range of degree of polymerization, monomeric substitution, species quantitation via mass-peak distribution, and composition of intermolecular bonds has been demonstrated . Furthermore, SI and SII thearubigins have been analyzed by MALDI-TOF-MS by Menet et al.[14] employing matrices 2′,4′,6′-trihydroxyacetophenone and α-cyano-4-hydroxycinnamic acid (**A**), respectively. Spectra were obtained in the positive-ion detection mode. Theaflavin (TF) standards were initially analyzed for their MALDI-TOF-MS characteristics. The main fragmentation patterns involved cleavage of the gallate ester functions and retro-Diels−Alder (RDA) fission of the C-ring. On the basis of the molecular ions and fragmentation ions observed in the mass spectra of SII TRs, Menet et al. hypothesized that structural fragments derived from catechins, such as a proanthocyanidin or a theasinensin, comprise SII TRs and suggested that formation of SII TRs consisted, in part, of the oxidative coupling between a 3,4,5-trihydroxyl moiety of the gallate esters of a dimer (theasinensin- or proanthocyanidin-type) and a catechin derivative ((−)-epigallocatechin gallate, (−)-epicatechin gallate, (−)-gallocatechin, (−)-epigallocatechin, (−)-epicatechin, (+)-catechin, (−)-catechin gallate, or (−)-gallocatechin gallate). MALDI-TOF-MS studies on dietary polyphenols have been reviewed by Reed.[15] The majority of studies on complex mixtures of dietary polyphenols have utilized α-cyano-4-hydroxycinnamic acid (**A**) as the matrix with a few exceptions utilizing trans-3-indolacrylic acid (**C**).[10,11,13,15]

■ MATERIALS AND METHODS

Chemicals and Reagents. All chemicals and reagents, including matrices, were purchased from Sigma-Aldrich and Fluka and were of the highest purity available. Commercial black tea samples (internally referred to as the selection of world teas) were kindly provided by Unilever, Colworth Science Park, U.K.

Preparation of Thearubigins. For all of our studies we chose a method based on that introduced by Roberts[3] wherein the majority of phenols in a black tea brew precipitate after complexation to added caffeine, leaving most nonphenols in solution. 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 Whatman no. 4 filter paper to remove the leaves, and the remaining brew was allowed to cool to room temperature. Caffeine, sufficient to achieve a concentration of 20 mM, was added to the brew with stirring to ensure dissolution. The brew was allowed to stand at 4 °C for 2 h and was then centrifuged at 23300g for 20 min. The resulting precipitate was recovered and suspended in boiling water and then 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 and SI and SIa thearubigins) 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 then freeze-dried. The freeze-dried material (SII CTR fraction) was stored at −20 °C, allowed to thaw, and reconstituted as required. SII CTRs were obtained as light-brown to rust-brown fluffy powders in

yields ranging from 5 to 10% (w/w) (see Table 1 of the Supporting Information). Saturated aqueous solutions of SII CTRs were orange to reddish-brown in color.

MALDI-TOF Mass Spectrometry. MALDI-TOF spectra were acquired on an Autoflex II MALDI-TOF-TOF mass spectrometer (Bruker Daltonics) equipped with a 337 nm nitrogen laser. The instrument was operated in the reflector mode: source, 19.08 kV; lens, 8.23 kV; and reflector, 20.06 kV, using an optimized ion extraction delay time of 80 ns. The laser frequency was set at 25 Hz with 50 laser shots per acquisition. The laser power was optimized to obtain a strong analyte signal, individually for each experiment, in the range from 38 to 45%. The matrix suppression mode was set to deflection, up to 400 amu. Spectra were obtained by summing, on average, 400 laser shots (with a maximum of 850). Spectra were acquired in the mass range 500−2500 amu with a detector gain of 3.5×, an optimized electronic gain of between 50 and 100 mV, and a sample rate of 2.00 GS/s.

The instrument was externally calibrated in the enhanced quadratic calibration mode prior to acquisition using a peptide tune-mix sample (Bruker Daltonics) followed by internal calibration after acquisition with a mixture of three well-known tea polyphenols, theaflavin (**9**), theaflavin-3-monogallate (**10**), and theaflavin-3,3′-digallate (**11**) (see Chart 1), and tannic acid. Spectra were processed using Data Analysis 4.0 software (Bruker Daltonics).

Matrix Preparation and Application. α-Cyano-4-hydroxycinnamic acid (**A**), 3-aminoquinoline (**B**), trans-3-indolacrylic acid (**C**), 2,5-dihydroxybenzoic acid (**D**), 3-hydroxypicolinic acid (**E**), 3′,4′,5′-trihydroxyacetophenone (**F**), and 2′,5′-dihydroxyacetophenone (**G**) were prepared as saturated acetone solutions. A volume of 1 μL of saturated matrix solution was drawn with a Gilson pipet over an anchor, and the matrix crystals were allowed to air-dry. trans-Stilbene (**H**) and anthracene (**I**) were prepared as saturated chloroform solutions. A volume of 2 μL of saturated matrix solution was applied with a Gilson pipet to a ground stainless steel target spot, and the matrix crystals were allowed to air-dry.

MALDI Targets. Matrices **A−G** were applied to an MPT AnchorChip 600-384 target (Bruker Daltonics). Matrices **H** and **I** were applied to a ground stainless steel MALDI target (Bruker Daltonics).

Sample Preparation and Application. Theaflavins **9−11**, tannic acid, or SII CTRs (1.0 mg) were dissolved in MeOH (500 μL). One microliter of this MeOH solution was dissolved in 50 μL of acetone. For application to the MPT AnchorChip target, 2 μL of the acetone solution was applied to the matrix crystals on each anchor. The sample was allowed to air-dry. For application to the ground stainless steel target, 1 μL of the acetone solution was applied to the matrix crystals on each ground stainless steel target spot. The sample was allowed to air-dry.

■ RESULTS AND DISCUSSION

Isolation and Purification of SII CTRs. Most studies of thearubigins have utilized a single type of black tea. For this study the world tea collection supplied by Unilever (see Table 1 of the Supporting Information) was investigated because it better represents the types of teas commercially produced and encompasses geographic variations in agricultural practice and processing. The nomenclature of the SII CTRs used herein is consistent with the nomenclature used in our previous papers. Many processes have been reported for the isolation and fractionation of thearubigins, but in the absence of analytical methods well suited to their characterization, it has not been possible to ascertain how composition varies with method of preparation.

Fractionation efficiency was assessed by HPLC using a modification of a published HPLC method.[16,17] In each case the HPLC chromatogram, with UV monitoring at 400 nm, showed a broad unresolved hump with a retention time in the
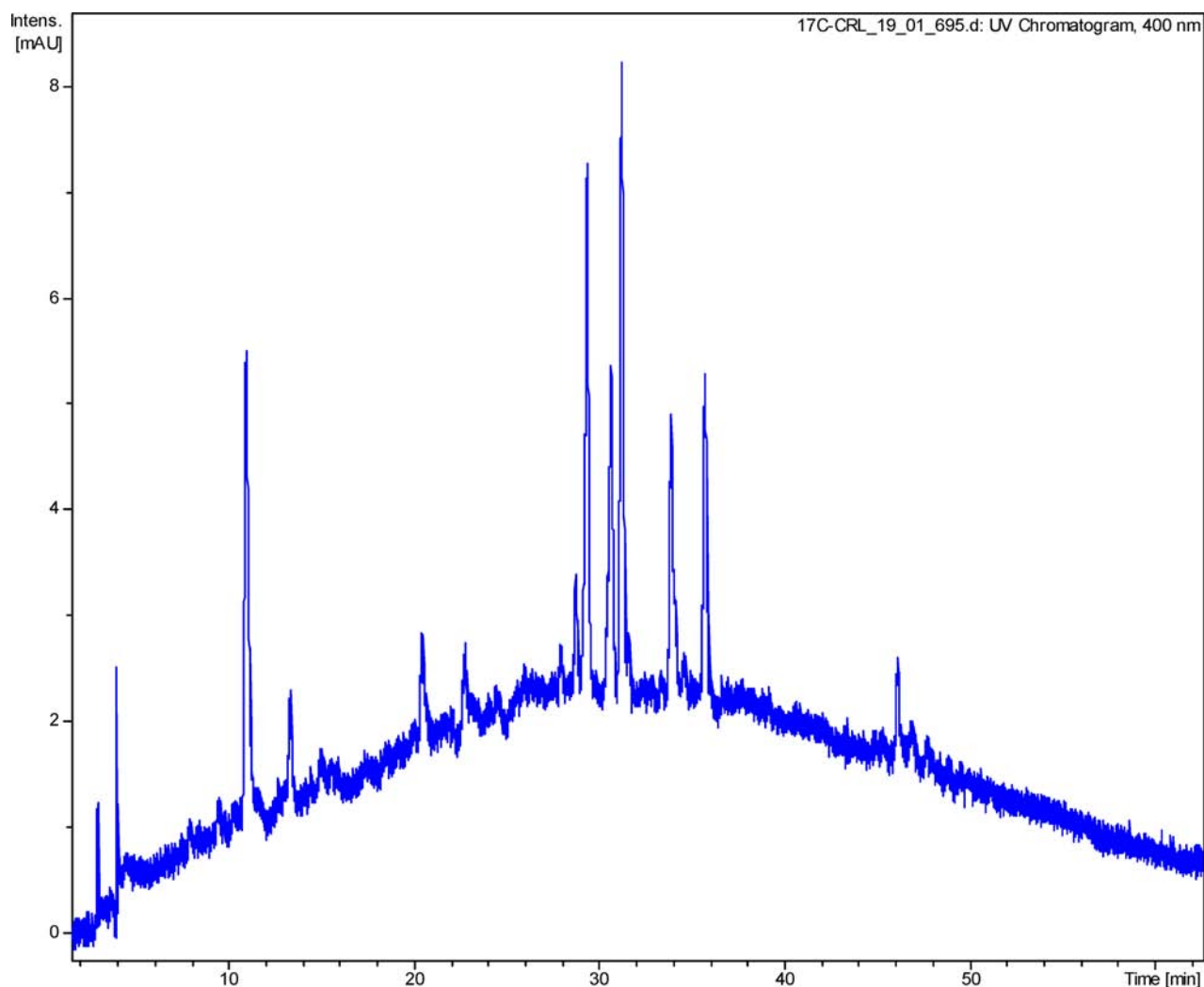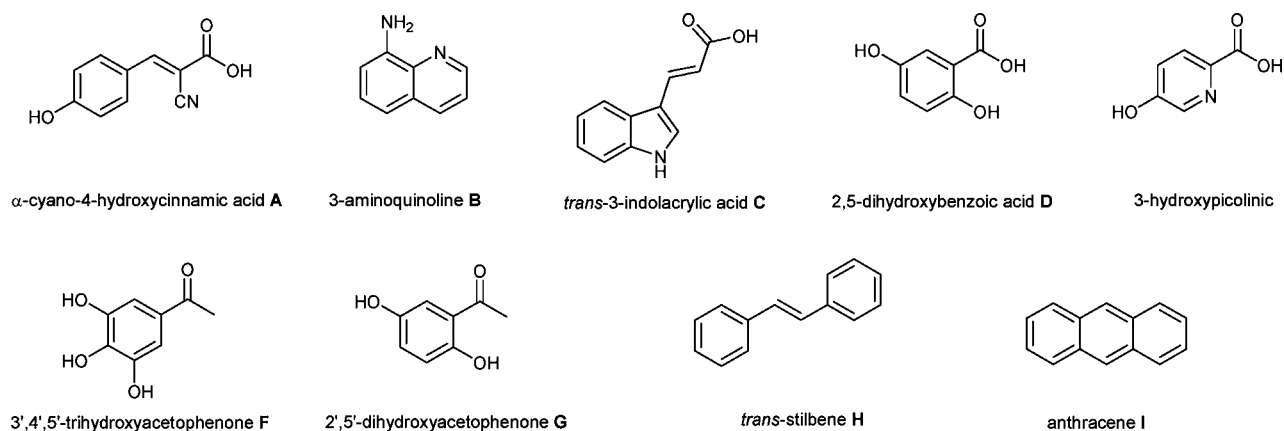
Figure 1. HPLC chromatogram of SII CTR fraction TR II: UV at 400 nm showing the thearubigin hump.

Chart 2. Structures of Compounds (A–I) Tested as MALDI Matrices

α-cyano-4-hydroxycinnamic acid **A**   3-aminoquinoline **B**   *trans*-3-indolacrylic acid **C**   2,5-dihydroxybenzoic acid **D**   3-hydroxypicolinic

3',4',5'-trihydroxyacetophenone **F**   2',5'-dihydroxyacetophenone **G**   *trans*-stilbene **H**   anthracene **I**

range from 5 to 25 min with near Gaussian shape. This hump has been generically termed the thearubigin hump and is characteristic of this type of material. Monitoring at 280 and 360 nm indicated that only small amounts of catechins, caffeine, flavonol glycosides, and theaflavins were present, appearing as small sharp peaks "floating" on the hump (Figure 1). These

residual contaminants were present at <10% of the concentrations originally present in the whole black tea brew.

The choice of matrix, which should be able to induce ionization of the analyte, is particularly crucial to the results obtained from a MALDI-TOF-MS experiment and requires careful consideration and optimization. Important factors that need to be optimized and considered are preparation and

application of the analyte, which may include the addition of metal salts to enhance the MALDI signal; selection of suitable mass calibration methods; linear or reflector mode; ion extraction delay and, if utilized, its length; laser power; polarity of extraction field; selection of "sweet spots"; and correct interpretation of spectra.

We started our investigation by screening a selection of MALDI matrices reported in the literature and two novel UV-MALDI matrices; *trans*-stilbene (**H**) and anthracene (**I**) (in both positive- and negative-ion detection modes). To the best of our knowledge no systematic study of matrices suitable for the investigation of complex dietary polyphenols has been carried out. It appears that most workers have been rather fortunate when selecting a matrix and were content with the results obtained. Chart 2 shows compounds we tested as matrices for SII CTRs. As test analytes we chose tannic acid, the main constituent of which is gallotannin, which possesses some chemical and structural similarities to black tea polyphenols (e.g., carbohydrate, gallate ester and phenol groups); and a mixture of isolated theaflavins (**9−11**) known to be present in black tea and SII CTR sample TR IV.

From inspection of the spectra it becomes immediately obvious that large differences in spectral quality exist between the different matrices employed. However, it is difficult to quantify these differences. MALDI-TOF mass spectrometry is subjective and depends on the individual preferences of the analyst, with matrix and analyte preparation appearing to retain an empirical approach. Even within a single MALDI matrix preparation, large differences in spectral quality exist between various crystalline regions of an anchor. We therefore opted for the following approach: at each anchor multiple spectra were acquired and summed, with variation of laser power and shot location, within the anchor region. The sweet spot effect was observed across the various crystalline regions, and sweet spots were searched when analyte signals were low. An energy transfer-induced disproportionation (ETID) ionization model has recently been disclosed to explain the sweet spot effect observed for some common UV-MALDI matrices, including α-cyano-4-hydroxycinnamic acid (**A**), 2,5-dihydroxybenzoic acid (**D**), and 3-hydroxypicolinic acid (**E**).[8]

The "best" spectrum was taken as a measure for spectral quality. For the tannic acid samples, the overall signal-to-noise ratio (S/N) of the 10 most intense peaks was taken as a direct measure for spectral quality. For the thearubigin sample, the number of peaks detected with a S/N threshold above 10 was taken as a measure of spectral quality (Table 1). The spectra were then classified into three categories; poor (−), moderate (0), and good (+). Table 2 in the Supporting Information summarizes the results; α-cyano-4-hydroxycinnamic acid (**A**) gave only mediocre performance in this test. The best matrices were found to be 3-aminoquinoline (**B**), 3′,4′,5′-trihydroxyacetophenone (**F**), and 2′,5′-dihydroxyacetophenone (**G**) in the negative-ion detection mode. Figure 2 shows two selected negative-ion detection mode mass spectra of TR XII utilizing α-cyano-4-hydroxycinnamic acid (**A**) as matrix compared with using 3-aminoquinoline (**B**) as matrix.

Selection of MALDI Target. AnchorChip targets are equipped with hydrophilic "anchors" surrounded by a hydrophobic coating. After application, the analyte is concentrated toward the center of the anchor and thus reduces the search area for sweet spots. AnchorChip targets are also equipped with additional anchors, in close proximity to the analyte anchor, for improved external calibration. In our study, we found that

Table 1. Number of Peaks Observed for Different Thearubigin Fractions in Negative- and Positive-Ion Mode Detection Utilizing 3′,4′,5′-Trihydroxyacetophenone (F) as Matrix (850 Summed Laser Shots)

TR	black tea leaf description	no. of peaks in negative-ion mode		no. of peaks in positive-ion mode	
		S/N > 3	S/N > 10	S/N > 3	S/N > 10
I	Kenya	3120	215	840	89
II	Darjeeling	2988	301	900	94
III	Lipton Blend	2876	298	756	71
IV	Vietnam Dust	3798	416	476	55
V	Turkish	2654	191	978	103
VI	Tiger Hill	4106	402	830	105
VII	Kenyan BP1	4498	407	904	97
VIII	Java Broken	3698	375	1010	162
IX	Indian BB21	3973	365	398	122
X	Darjeeling White Leaf	2078	209	790	91
XI	Ceylon UVA	4193	416	879	71
XII	Ceylon Standard EBOP	4538	399	804	100
XIII	Ceylon GMD	3619	374	573	47
XIV	Assam	3219	400	796	89
XV	Argentine BOP	4396	270	976	104

Anchorchip technology provided ease of matrix-sample preparation when matrices **A−G** were prepared as saturated acetone solutions. An attempt to apply a saturated acetone solution of 1,8,9-trihydroxyanthracene (dithranol) matrix to anchors failed, and this matrix was not further investigated during the present study. *trans*-Stilbene (**H**) and anthracene (**I**), both hydrophobic in nature, could be applied only to a conventional, ground stainless steel MALDI target. Anthracene (**I**) exhibited good application to the target when prepared as a saturated chloroform, *i*-PrOH, or diethyl ether solution, and *trans*-stilbene (**H**) exhibited good application to the target when prepared as a saturated chloroform solution.

MALDI-TOF Mass Spectra. As discussed above, having fulfilled the relevant criteria, 3′,4′,5′-trihydroxyacetophenone (**F**) was selected as the optimal matrix, faring only slightly better than 2′,5′-dihydroxyacetophenone (**G**), for acquiring the mass spectra of all SII CTRs in the negative- and positive-ion detection modes. The negative-ion mode spectra were, on average, of better spectral quality compared with the positive-ion mode spectra. Five representative spectra are shown in Figure 3. It is worth noting that both matrices, 3′,4′,5′-trihydroxyacetophenone (**F**) and 2′,5′-dihydroxyacetophenone (**G**), yielding the strongest analyte signals in the negative-ion detection mode, are *m*-, and *o*-hydroxy-substituted acetophenones, respectively. This suggests that a matrix containing a hydroxy-substituted, non-carboxylic acid-type carbonyl moiety is efficient for thearubigin ionization under positive-polarity extraction voltage conditions. However, a discussion of a proposed mechanism, based on hydroxyl substitution and carbonyl interaction, is beyond the scope of the current paper.

Calibration was carried out in two steps. First, an external calibration using a peptide reference standard was carried out, followed by an internal calibration using a selection of six compounds within the black tea samples. The presence of all six well-characterized reference compounds in the SII CTRs used for calibration was previously established using HPLC-tandem-MS, HPLC-UV, and HPLC-ESI-TOF-MS and by comparison
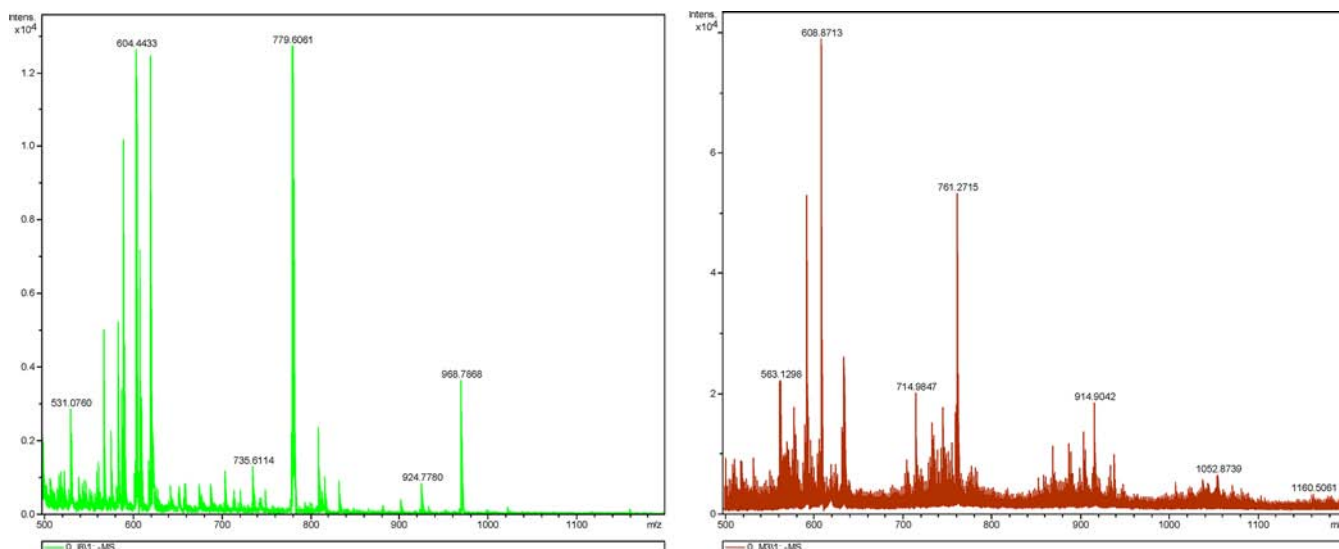
Figure 2. Negative-ion mode MALDI-TOF mass spectra of TR XII utilizing α-cyano-4-hydroxycinnamic acid (**A**) as matrix (left) and 3-aminoquinoline (**B**) as matrix (right).
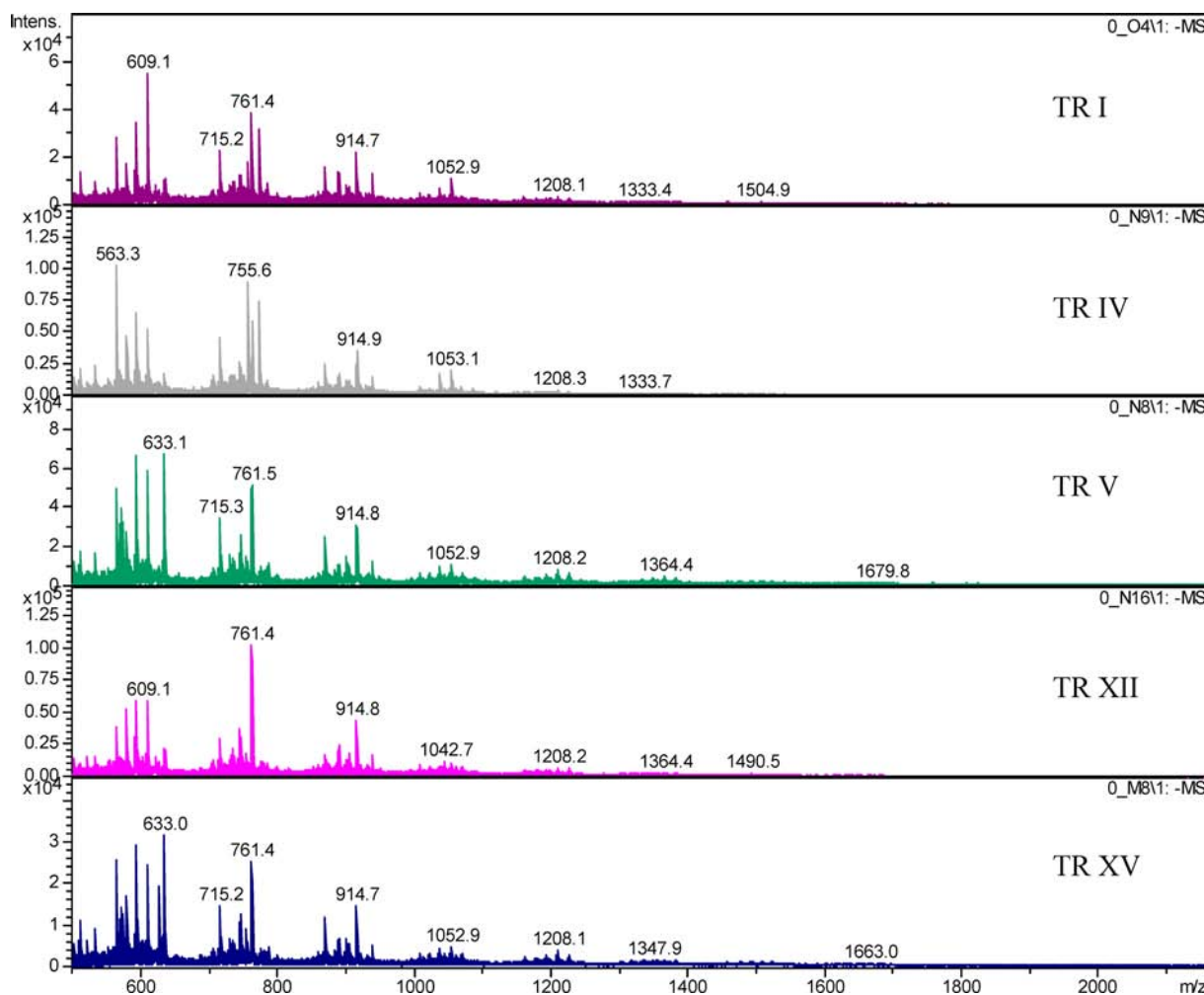

Figure 3. Five representative negative-ion mode MALDI-TOF mass spectra of TR I, TR IV, TR V, TR XII, and TR XV utilizing 3′,4′,5′-trihydroxyacetophenone (**F**) as matrix.

with authentic reference materials of the standards. The results of the calibration were in general disappointing with standard deviations for all samples above 10 ppm and an average

standard deviation of 33 ppm over all 15 samples. Even reference calibration materials containing 10 known and previously purified polyphenolic black tea standards resulted

in calibration curves with standard deviations of >10 ppm, despite all efforts to improve this. The validity of this type of calibration needs careful consideration and will be revisited below when redox artifacts are discussed.

With calibration results of this type, a reliable assignment of molecular formula from the "high-resolution mass data" is problematic, and we therefore abstained from obtaining molecular formula data from this data set. It should be kept in mind that the peaks observed in the MALDI spectra largely coincide, within an error of 0.0001 amu, with those of LC-ESI-MS and LC-APCI-MS, and direct infusion ESI-FTICR and ESI-TOF data and these measurements allow a more reliable determination of molecular formulas. Furthermore, it is worth noting that, in most literature, MALDI-TOF data of polyphenols are quoted to only one decimal place,[15] with other authors potentially suffering from similar problems as described here. One might be tempted to argue that these poor calibration results are a direct consequence of the complexity of the SII CTRs, with several thousand components being present, resulting in overlapping signals shifting the measured mass value. However, direct infusion ESI-TOF and APCI-TOF data of identical samples, with all instruments operating at an experimentally determined resolution of around 17000 at m/z 300 after internal calibration, dictate a different explanation is needed. The ESI-TOF and APCI-TOF data give standard deviations of the calibration curve of <10 ppm (7.1 ppm on average over all SII CTRs for ESI-TOF data, for example), much superior to the MALDI-TOF data, suggesting that low mass accuracy in this case appears to be a MALDI-specific problem for this class of compounds.

For each mass spectrum the number of peaks was determined for thresholds of two signal-to-noise ratios: S/N > 10 and S/N > 3. From these data it becomes apparent that the number of peaks present at the smaller S/N threshold is by an order of magnitude larger when compared with the higher level. A detailed discussion of this observation follows in the discussion on spectral noise. For S/N values of 10, between 300 and 1000 peaks could be detected in each MALDI spectrum, whereas for a S/N ratio of 3, between 1000 and 4500 peaks were detected.

The spectra of SII CTRs from the 15 world teas are all characterized by the appearance of mass clusters centered at m/z 610, 760, 910, 1060, 1210, 1360, 1500, and 1650 (see Figure 3). Two spectra show further mass clusters at m/z 1900 and 2050, with the highest mass with a S/N > 3 detected at m/z 2101 (data not shown). The spacing of these mass clusters corresponds to one gallic acid ester unit (m/z 152) or one gallocatechin unit (m/z 305), indicating that the majority of peaks observed can be assigned as oligomers of catechins and their gallate esters **1−6**. A rough assignment is given in Table 2. These observations indicate that the SII CTRs are oligomers not exceeding 2100 amu. These observations are in agreement with ESI-FTICR data, where a plot of number of carbons in a SII CTR component versus its sum of intensities or numbers of compounds with that molecular formula revealed clear maxima, which were interpreted in terms of gallated-catechin oligomerization.

When one zooms into the mass clusters (see Figure 4), the following two features are apparent: (i) starting from a major peak, for example, theaflavin **9** at m/z 563, further larger peaks appear at mass intervals of +16 amu (most likely addition of an oxygen atom), for example, at m/z 579, 595, 611, and 623, or starting at m/z 715 for a theaflavin monogallate (such as **10**)

Table 2. Assignments of Mass Clusters Observed in Negative-Ion Mode MALDI-TOF-MS of Thearubigins

cluster	cluster m/z center	assignments
1	610	dimer of catechin
2	760	dimer of one gallated catechin and one catechin
3	910	dimer of two gallated catechins or trimer of three catechins
4	1060	trimer of two catechins and one gallated catechin
5	1210	tetramer of four catechins or trimer of two gallated catechins and one catechin
6	1360	tetramer of three catechins and one gallated catechin
7	1500	pentamer of five catechins or tetramer of two catechins and two gallated catechins
8	1650	pentamer of four catechins and one gallated catechin or tetramer of one catechin and three gallated catechins
9	1800	hexamer of six catechins, tetramer of four gallated catechins, or pentamer of two gallated catechins and three catechins
10	1950	hexamer of five catechins and one gallated catechin
11	2100	heptamer of seven catechins, hexamer of two gallated catechins and four catechins, or pentamer of four gallated catechins and one catechin

the associated peaks are at m/z 731, 747, 763, 779, etc.; (ii) spaced between these series of peaks are masses that show mass increments of −2 amu (most likely −H_2), for example, 563, 561, 559, etc., indicating an oxidation reaction such as quinone formation. These observations are entirely consistent with the observations made in our previous work analyzing SII CTRs by FT-ICR-MS and support the structural hypothesis put forward in our previous paper.[1] This pattern is observed throughout the full mass range of m/z 500−1350.

In terms of peak assignment, it is worth reporting that the m/z ratios of the 20 most intense peaks roughly coincide with the m/z values of the peaks observed in the ESI-FTICR mass spectra including theaflavin, a theaflavin monogallate, theaflavin-3,3′-digallate, a theacitrin monogallate, and several flavonoid glycosides.[1] During ESI-FTICR-MS analysis, for 10 of the most intense peaks, tandem-LIFT MS experiments were carried out, and the MS^2 spectra confirmed the structure assignment. For all minor components, tandem MS experiments were attempted, but only poor-quality tandem MS spectra that do not merit interpretation were obtained.

Redox Artifacts in MALDI-TOF Mass Spectrometry. Close inspection of the MALDI-TOF mass spectra of the SII CTRs shows that in all mass regions there are clusters of peaks. Usually there are between 6 and 10 peaks present in the clusters, with adjacent signals being spaced by 2 amu. This spectral feature is present in all spectra, independent of the matrix employed or the sample analyzed. Inspection of original spectra available in the literature reveals that this feature is common for most complex polyphenols analyzed by MALDI-TOF mass spectrometry. The only spectra published that do not seem to show this phenomenon are line spectra or original spectra obviously subjected to smoothing routines or spectra showing broadened peaks. This phenomenon is largely absent in ESI-TOF mass spectra. Reed et al. have observed mass clusters, comprising −2 amu increment signals and predicted isotope pattern peaks, during MALDI-TOF-MS analysis of proanthocyanidins isolated from cranberries.[15] However, the −2 amu signals were attributed to real structural features of the analytes.

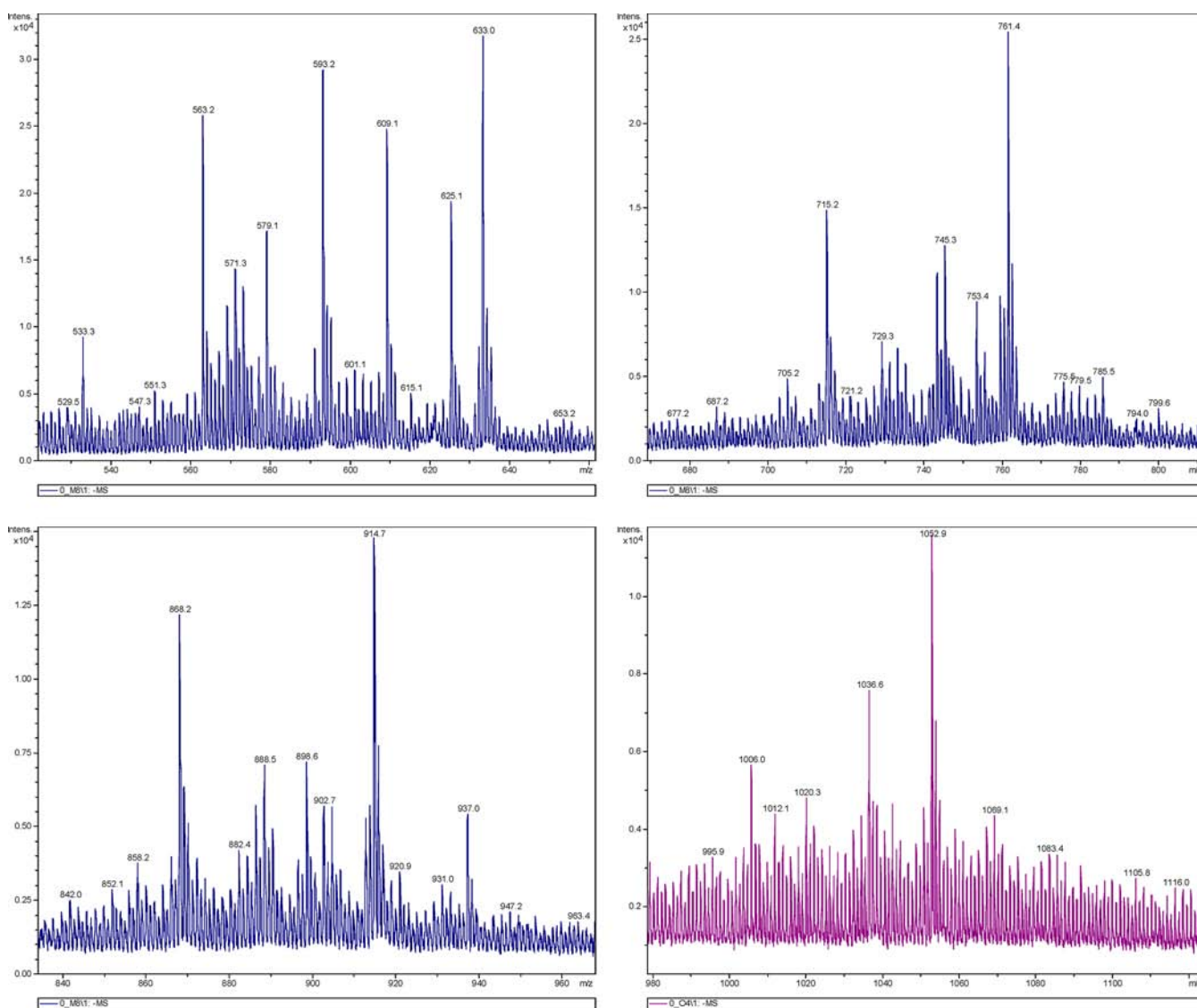The presence of these ion clusters has dramatic consequences for data analysis and interpretation: (i) The presence

Figure 4. Expanded regions of mass clusters of a negative-ion mode MALDI-TOF mass spectrum of TR XII utilizing 3′,4′,5′-trihydroxyacetophenone (**F**) as matrix.

of the clusters always prevents the use of isotopic pattern analysis for the determination of molecular formulas. An intense peak is always present at M + 2 amu, coinciding with the $^{13}C_2$ isotopic peak of the compound analyzed. (ii) Calibration needs to be carried out with great care. In cases in which our external calibration was not of the highest standard, the calibration routine of the software repeatedly assigned the wrong peak to the calibration mass defined in the calibration mass list. This misassignment of the first calibration mass is perpetuated through the whole spectrum, rendering high-resolution mass values meaningless. (iii) With external calibration only, calibration curve standard deviations above 200 ppm meant that no meaningful high-resolution mass values could be obtained.

The first question to be addressed is whether or not these spectral features represent the true composition of the sample or artifacts of the MALDI process. To address this question, a direct comparison of MALDI-TOF mass spectrometry data with ESI-TOF mass spectrometry data was undertaken for the tannic acid sample, a TF reference sample, and a selected thearubigin sample (TR XII) (see Figure 5). If these spectra are

directly compared, the following differences are apparent: (i) The MALDI-TOF mass spectrum shows a series peaks at m/z values above 1243 amu, which are absent in the ESI-TOF mass spectrum, although these compounds are apparent at lower m/z values, appearing as multiple-charged ions. (ii) The positions of the majority of mass peaks coincide with an average error of ±0.5 amu. (iii) The MALDI-TOF mass spectrum shows additional minor peaks; some of them with the aforementioned mass increment of −2 amu. These are largely absent in the ESI-TOF mass spectrum. (iv) The mass accuracy in the ESI-TOF mass spectrum is considerably better when compared with the MALDI-TOF mass spectrum, despite using identical calibration standards and methods.

One may argue that the two ionization mechanisms are fundamentally different with respect to the selectivity of ion formation. During the MALDI process some ions, for example, those in clusters, are formed preferentially, whereas the same do not ionize efficiently during ESI. However, it should be accepted that in both cases ionization efficiency depends on the physicochemical properties of the analytes and their ability to stabilize charges once formed. A case for the existence of
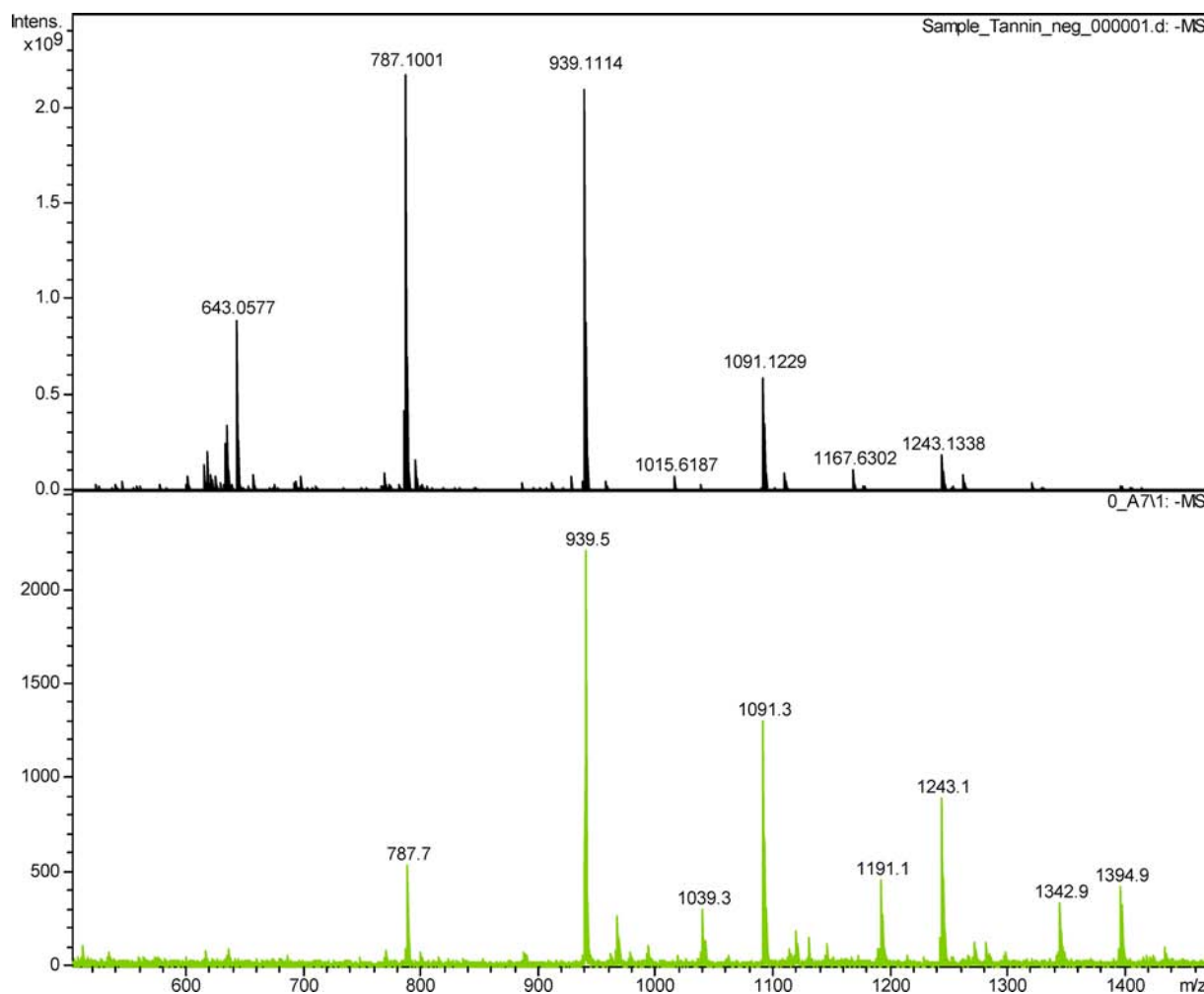
Figure 5. Comparison of a direct infusion negative-ion mode ESI-TOF spectrum (top) of a mixture of tannic acid and TR XII, and a negative-ion mode MALDI-TOF mass spectrum (bottom) of a mixture of tannic acid and TR XII utilizing 3′,4′,5′-trihydroxyacetophenone (**F**) as matrix. Both spectra were acquired at a resolution of 17000 at m/z 300.

MALDI-TOF mass spectrometry artifacts should be brought forward, which requires explanation and possible solutions.

Various ionization mechanisms have been proposed for the MALDI process, but no unified model exists. Mechanisms such as excited-state thermionic emission (a combined photo/thermal model),[18] energy pooling (a multicenter excitation mechanism),[7,19] ground-state proton transfer plume reactions,[20] shock waves,[21] and energy transfer-induced disproportionation (ETID),[8] including combinations of the above, have been proposed but remain uncertain and are currently under debate. A comprehensive review of MALDI MS has been published.[22]

One of the most frequently, and simplest, proposed ionization models, requiring absorption of only one photon, is excited-state proton transfer (ESPT).[23] The mechanism involves the absorption of a photon by a matrix molecule and subsequent promotion of the matrix molecule to an excited-state ($M + h\upsilon \rightarrow M^*$, where M represents a matrix molecule). Excitation enhances acidity, particularly phenolic protons, and results in the subsequent protonation of the analyte, which then leads to ion formation in the positive-ion detection mode ($M^* + A \rightarrow (M - H)^- + AH^+$, where A represents an analyte molecule). Additionally, a ground-state matrix molecule may accept the proton before the excited matrix molecule relaxes

($M^* + M \rightarrow (M - H)^- + MH^+$). By similar reasoning, in the negative-ion detection mode deprotonation of a matrix molecule excited state, followed by relaxation to the ground state and deprotonation of the analyte, produces a negative ion ($M^* - H^+ \rightarrow M^- + H^+ \rightarrow MH + A^-$). The observation that often negative and positive ions can be generated by the same matrix can be explained by a disproportionation mechanism (2 $M \rightarrow (MM)^* \rightarrow (M - H)^- + MH^+$); however, this representation is most likely oversimplified.

It is worth noting that matrices **A–G** (with the exception of 3-aminoquinoline (**B**) and *trans*-3-indolacrylic acid (**C**)) are polyphenolic in nature and are, therefore, in many respects, chemically indistinct from the analyte. In the present study their characteristics appear to significantly influence the MALDI process and ensuing spectral quality. Employing the ESPT model, it is uncertain whether the matrix or the analyte initially absorbs the photon and, thereafter, which species transfers a proton. Additionally, both matrix and analyte, once having transferred a proton in their excited states, become phenolate anions with an exceedingly more favorable oxidation potential compared with the corresponding phenols. These phenols, which participate as reducing agents for all other components present in the sample, can be easily oxidized. A redox-couple between phenol–phenolate and quinone gallate
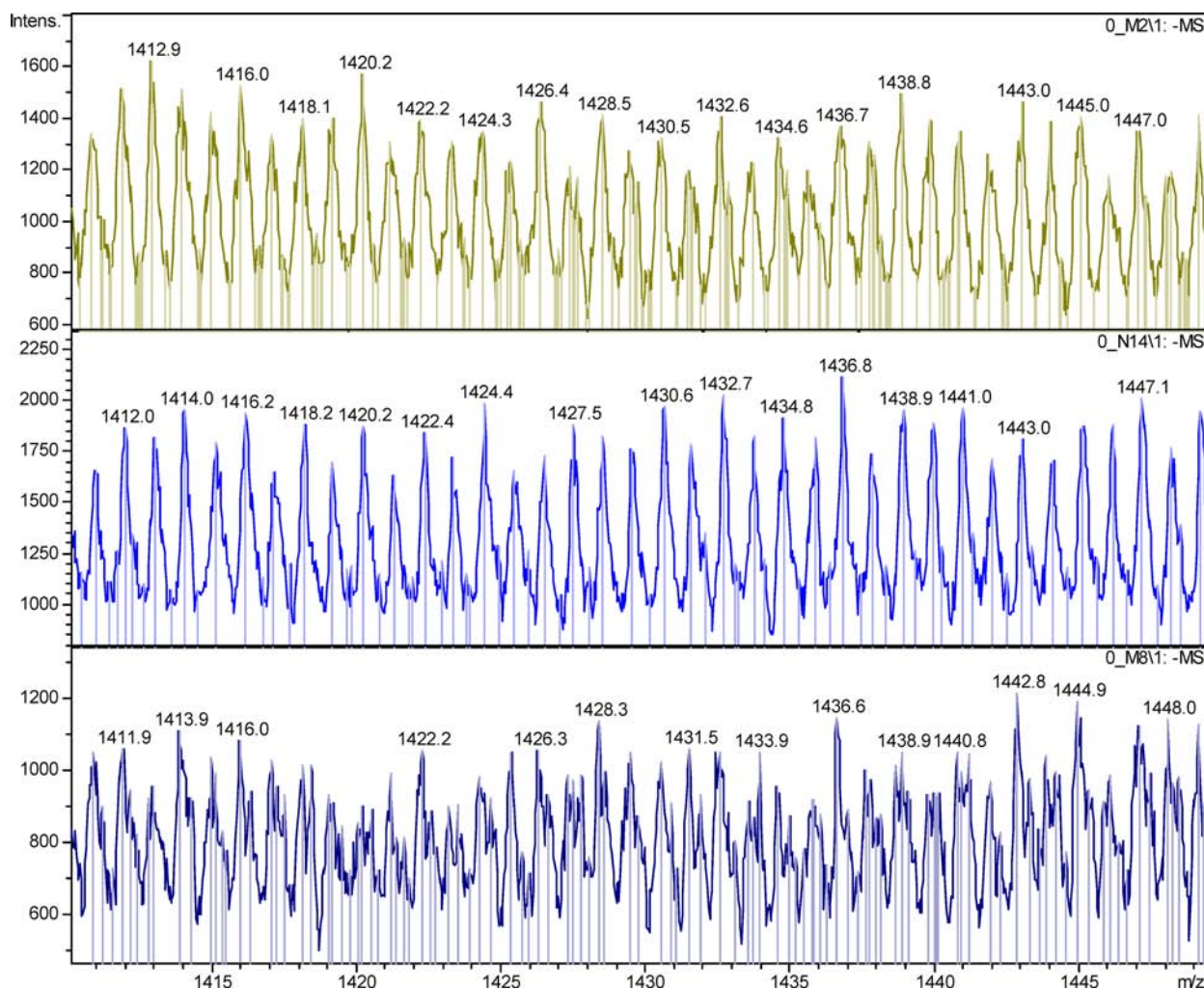
Figure 6. Expanded regions of the baseline of a negative-ion mode MALDI-TOF mass spectrum of TR IV, TR XII, and TR XIV utilizing 3′,4′,5′-trihydroxyacetophenone (**F**) as matrix.

ester is realized, involving matrix and analyte, most likely via a disproportionation-type mechanism.

Thearubigin polyphenols typically possess several oxidation steps, as shown, for example, by cyclic voltammetry, resulting in several redox steps, per analyte molecule, that are apparent in the MALDI-TOF mass spectra as redox artifact clusters.[24,25] For the MALDI matrices used in this work, and all other papers in the literature, these artifacts are present and must be taken into consideration during data interpretation; that is, one ion within a cluster is the structural basis for formation of the reduction and oxidation products, spaced by 2 amu, that constitute the cluster. This problem could be solved by the development of a "redox-silent" MALDI matrix, able to ionize complex polyphenols, but free from the disadvantage of artifact production.

We reasoned that such a matrix should possess the following characteristics: (i) it must possess a reasonable molar extinction coefficient at a wavelength of 337 nm (the laser irradiation wavelength); (ii) it must be chemically compatible with polyphenols, but (iii) it must be chemically different from polyphenols; and (iv) it should be nonbasic in negative-ion detection mode (positive-polarity extraction voltage) and nonacidic in positive-ion detection mode (negative-polarity extraction voltage). Accordingly, we selected two compounds fulfilling these criteria, *trans*-stilbene (**H**) and anthracene (**I**),

and tested their performance. Anthracene (**I**) has previously been identified, and utilized, as an electron-transfer MALDI matrix for the analysis of ferrocene derivatives; this work proposed that ionization of the analyte was induced by resonant two-photon ionization of the matrix, followed by electron transfer from the analyte to the matrix radical cation.[26] However, to the best of our knowledge, anthracene (**I**) has not been utilized as a UV-MALDI matrix to study polyphenols.

trans-Stilbene (**H**) demonstrated poor overall performance. Anthracene (**I**) yielded better quality spectra with a ∼50% overall reduction in the intensity of the +2 amu peaks for our test analytes and two selected thearubigin samples. However, the signal intensities were considerably reduced when compared with the use of 3′,4′,5′-trihydroxyacetophenone (**F**) as matrix.

Chemical Noise? Besides mass clusters, a second spectral feature requires consideration: at higher *m/z* values a periodic disturbance of the baseline is visible throughout all MALDI-TOF mass spectra (see Figure 6). At these points of maximum disturbance of the baseline, S/N ratios range from 2 to 3.5. This periodic function has a periodicity of 1 amu, starting around *m/z* 1000 and ending at 2000. If several spectra are compared, the position of the maxima are virtually identical. Hence, this baseline function may be a real feature of the spectra, and not chemical noise. The question is whether or not these are

structural features of SII CTRs or artifacts of the MALDI process. The presence of this periodic disturbance indicates the presence of redox clusters throughout a mass window of >1000 amu with at least one analyte hypothesized to being present every 2 amu. If one assumes that these spectral features are representative of the components of the SII CTRs, then it indicates the presence of another 2000−3000 components previously not observed in FTICR-MS data. It is, however, as well feasible that the periodic feature arises from noncovalent complexes formed from individual TR components, as shown previously by AFM and diffusion NMR methods.[27]

Accepting the hypothesis to explain the redox artifacts, one could assume that following the MALDI process large quantities of highly electrophilic quinone structures are generated in the presence of highly nucleophilic phenols. These could react to form a large variety of reaction products covering the whole mass range and explaining the periodic nature of the noise. As a consequence, great care must be taken when MALDI-TOF mass spectrometry data of SII CTRs are analyzed, and analysts should accept only the minimum of information that can be retrieved in an artifact-free form.

We have shown that MALDI-TOF mass spectrometry of polyphenols is particularly susceptible to artifact formation, especially when the material analyzed is a mixture as complex as the SII CTRs. The extent of artifact formation is greatly influenced by the choice of matrix and, ideally, the matrix should be redox-silent. None of the matrices tested was perfect: 3′,4′,5′-trihydroxyacetophenone (**F**) provided the best compromise between signal intensity and redox artifact formation, and it is possible that a *m*- or *o*-hydroxy-substituted carbonyl moiety may be a prerequisite for successful thearubigin ionization.

The MALDI-TOF mass spectra of the SII CTRs isolated from the 15 world teas revealed up to 4500 peaks in a mass range from m/z 500 to 2100, thus demonstrating the presence of even more distinct components than previously observed. Three sets of mass clusters were observed: (i) differing by ∼150 and/or ∼300 amu; (ii) separated by 16 amu; and (iii) separated by ±2 amu. These observations are consistent with our previous conclusion that SII CTRs are polyhydroxylated oligomers (rather than polymers) of catechins and catechin gallates in redox equilibrium with their quinone counterparts. Signals corresponding to many small-molecule polyphenols, previously reported in black tea, were also observed.[4]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional tables and figure. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: n.kuhnert@jacobs-university.de; Phone: 0049 421 200 3120.

Notes
The authors declare no competing financial interest.

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