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Analysis of black tea thearubigins from six different commercial teas by ESI FT-ICR MS

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Introduction

Thearubigins are the most abundant group of phenolic pigments found in black tea accounting for an estimated 60 % of the solids in a typical black tea infusion. Up to now the chemical nature of the thearubigins remains largely unresolved if not mysterious.
Thearubigins isolated from six commercial black teas have for the first time been analyzed by Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometry (MS). Up to 9 000 mass spectral peaks have been detected per sample using the ultra-high resolving power of FT-ICR MS. Due to the high mass accuracy better than 1 ppm molecular formulas assigned to up to 1500 compounds. More than 50 compounds have been named by comparison to the available literature. Not allowing for isomers, these thearubigin samples contain at least 2500 compounds, thus explaining the inability to resolve individual compounds chromatographically.
Data interpretation strategies developed for petrolomic studies (van Krevelen and Kendrick analyses) have been applied to black tea. A novel software program and a protocol have been developed to refine these procedures for the investigation of polyphenols. Using homologous series analysis oxygenation has been identified arising by nucleophilic addition of water to aromatic CH groups as a key feature. A series of reaction schemes have been developed linking known precursors with plausible products that match the available MS data. The accuracy of these predicted structures will be assessed critically by ion trap MS procedures.

Methods

Thearubigins were isolated from black tea in a simple method introduced by Roberts [1] in which the major pigmented polyphenols precipitate after complexation with added caffeine leaving most non-phenols in solution. The precipitate was redissolved in hot water and the thearubigins recovered as an aqueous solution after ethyl acetate extraction to remove the theaflavins and chloroform extraction to remove the caffeine. After freeze-drying, the thearubigins from the 15 black teas were obtained as light brown to rust brown fluffy powders in yields ranging from 5 % to 10 %. For FT-ICR-MS analysis the thearubigins were reconstituted in 1:1 water methanol at a concentration of 2 mg/ml and analyzed with an apex ultra 9.4 T FT-ICR instrument (Bruker Daltonics Inc., Billerica, USA) by direct infusion measurements in electrospray negative ion mode.

[1] Roberts, E. A. H., Economic importance of flavanoid substances: tea fermentation, in *Chemistry of Flavanoid Compounds* ed. Geissmann, T. A., Pergamon, Oxford **1962**, 468-512.

Figure 1:

Three dimensional van Krevelen plot (elemental ratio plot) showing the O/C ratio versus the H/C ratio with intensities of all assigned pseudo-molecular ions color coded in third dimension (key: the darker the more intense) for thearubigin fraction from Vietnam Dust black tea in negative ion mode.

The majority of data points lie within a region typical for characterized black and green tea polyphenols and oxidized polyphenols (data points shifted to lower H/C ratio and higher O/C ratio).

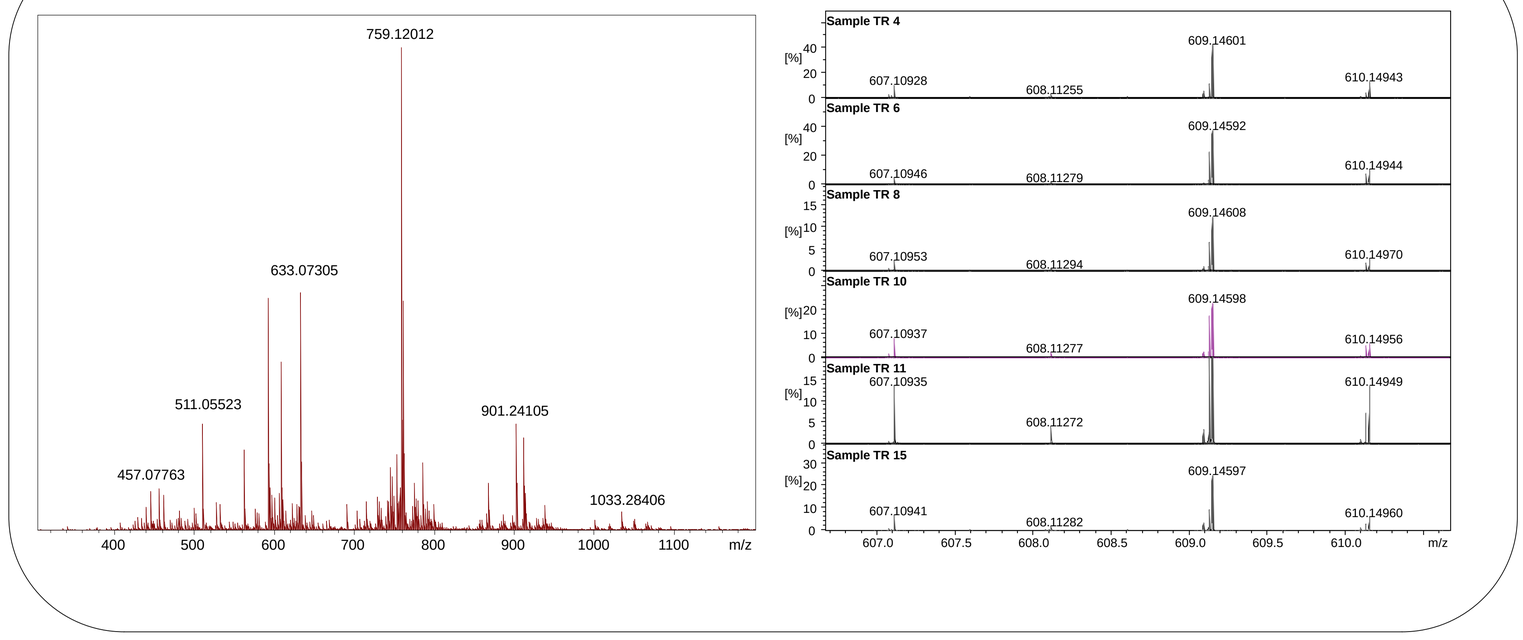


Figure 2:

FT-ICR mass spectrum of a thearubigin sample from Vietnam Dust black tea (left) and expanded region around m/z 609 of all six thearubigin samples (TR4 - TR 15) (right).

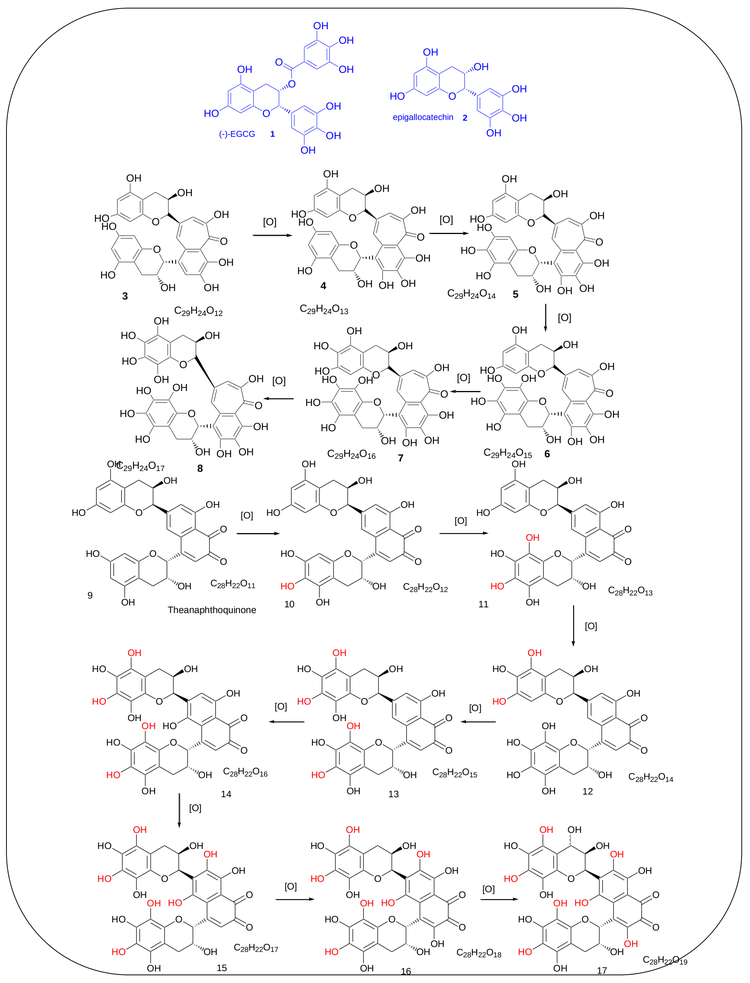
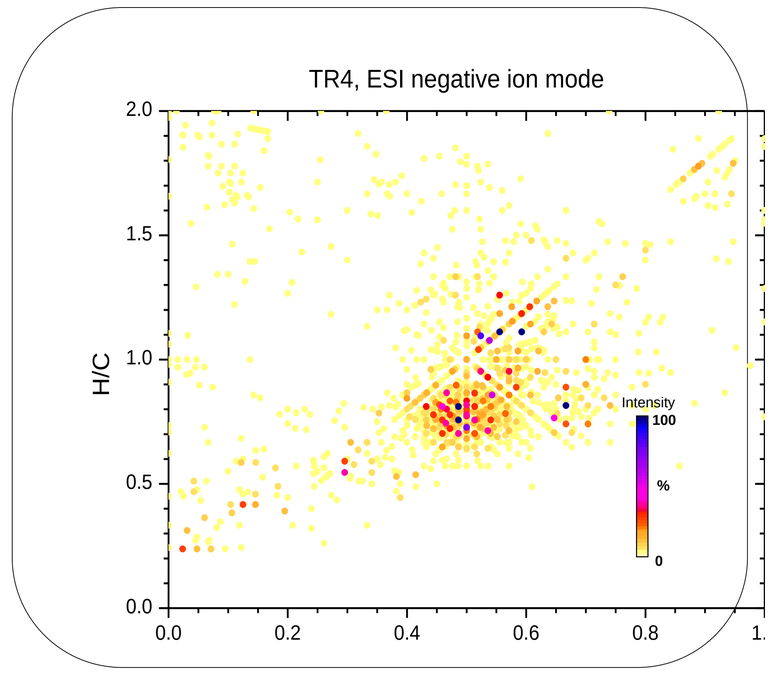


Figure 3:

Structures of green tea flavanoids EGCG **1** and epigallocatechin **2** along with proposed oxidation sequences forming polyhydroxy-theaflavins **3-8** and polyquinone-theanaphthoquinones **9-16**. Molecular formulas were determined from ESI FT-ICR-MS data.



Results

From the 9 000 resolved peaks in the experimental FT-ICR mass spectra we assigned around 1 400 molecular formulas with a mass accuracy below 1 ppm. From these 1400 molecular formulas around 50 molecular formulas were consistent with black tea polyphenols already described in the literature (around 80 have been described so far) and were tentatively assigned to the structures reported.

For structure assignment of the remaining 1350 molecular formulas a combination of various strategies was applied. A combination of van Krevelen analysis, Kendrick analysis, DBE vs. H/C plot and DBE vs. number of carbon plots revealed the presence of homologous series of compounds within the sample and provided molecular formula parameters that allowed rough classification of the compound classes present. From these findings a novel data search routine termed **H**omologous **S**eries **A**nalysis (HSA) was implemented that allowed the list of molecular formulas determined to be searched for series of structural increments.

These HSA searches provided the following hypothesis for the chemistry behind tea fermentation and the formation of the thearubigins. Green tea flavanoids such as EGCG **1** and epigallocatechin **2** are oxidatively oligomerized to form initially dimeric structures of the theaflavin, theanaphthoquinone, theacitrin and theasinensin type. These can be further oxidatively coupled to other monomeric green tea flavanoids. The enormous molecular diversity of black tea thearubigins is, however, achieved by successive addition of water to ortho-quinone type structures derived from the former flavanoid B-ring. Series of compounds eg. theaflavins **3-8** are formed by this oxidative hydration. Furthermore aromatic 1,2 diol moieties are in equilibrium with their oxidized quinone counterparts shown for theanaphthoquinones **9-16**. All regioisomers shown were randomly chosen.

Summary

➢A structural model for thearubigin formation has been developed based on experimental FT-ICR-MS data and novel data interpretation strategies.

Conclusions

ESI FT-ICR-MS provides a powerful tool for the analysis and structure elucidation of extremely complex dietary mixtures such as black tea thearubigins, which are several orders of magnitude too complex to be amenable to chromatographic analysis.

ESI FT-ICR MS