学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

氏名：　　　三井　一寿
Name

学位論文題目：
Title of Dissertation

The in-depth study of aroma compounds formed during tobacco combustion *via* Maillard reaction and/or caramelization
（シガレット燃焼過程でのメイラード反応およびカラメル化反応による加熱香気成分生成に関する研究）

学位論文要約：
Dissertation Summary

The aroma of foods is the most important sensory attribute affecting the acceptance and preference of consumers. It is widely accepted that the olfactory perceptions were caused by volatile or semi-volatile substances and depends in part on the aroma compounds present in the raw material and in part on how the food was prepared (thermally treated). Thermal treatment of foods results in desired and undesired effects which all derive from the chemical reactions, namely Maillard reaction, caramelization and to a minor extent, lipid oxidation. Through the Maillard reaction, together with caramelization, a large number and various quantities of compounds are generated. Several of these formed products play an important role in the aroma and taste of foods and consumer products. However, it's a complicated process which consists of many reactions and still it has not been clarified completely. Therefore the efforts for its understanding and control are of utmost importance to support the development of food industries. Beside the relevance in the applicative fields of food science, a deep understanding of the reaction mechanisms are essential for fundamental biological issue such as those related to the ingestion of these products.

This dissertation demonstrated some of the investigation designed to lead to an increased understanding of the chemistry of the Maillard reaction and caramelization for real-life samples, based on recent analytical developments. The novel analytical techniques was developed and applied to precursor screening, intermediate monitoring and trace level of aroma compounds detection, formed during tobacco combustion as one of the highly complex real-life sample, to clarify the influence on cigarette smoke aroma properties.

In chapter 1, the Maillard reaction and caramelization mechanism for the aroma compounds formed by thermal treatment of raw food and the practical case in daily food processing were summarized. In view of the large numbers of stages and the sensitivity of each stage in the reaction to changes in the conditions, it is very complicated to investigate and control the whole Maillard reaction and caramelization. The extent of, and the pathway for, change are controlled by many factors. These factors could be distinguished as those relating to the reactant composition, e.g., sugar type, type of amino acid, sugar and amino acid ratio and external factors such as moisture level, temperature, pH and so on. Additionally, traditional gas chromatography–mass spectrometry (GC–MS) and related novel analytical techniques, multi-dimensional GC, analytical pyrolysis and modern omics approach, of volatile and semi-volatile aroma compounds were reviewed.

In chapter 2, analytical method for the aroma precursor compounds affecting the cigarette smoke aroma formed during tobacco combustion was discussed. Analytical pyrolysis combined with GC–MS (Py-GC–MS) is an effective technique for studying thermally treated natural products and processes like cigarette smoking. Direct pyrolysis of tobacco leaf results in a very complex mixture of organic compounds containing volatile, semi-volatile and non-volatile compounds. Consequently, detecting and identifying the main cigarette smoke aroma compounds and their formation pathways are very difficult.

In this study, an innovative workflow that can elucidate the aroma precursor compounds in tobacco leaf was presented using a recently developed resistive heating pyrolysis device, namely a modified thermal desorption system (TDU-Pyro, Gerstel GmbH, Mülheim an der Ruhr, Germany). The workflow consisted of LC fractionation of a tobacco leaf extract, recombination of fractions in a fraction omission approach, Py-GC–MS and multivariate chemometric data analyses. Based on previous studies, an aqueous tobacco extraction was selected to demonstrate the importance of polar constituents such as carbohydrates and/or amino acids on the formation of Maillard reaction and caramelization aroma compounds. In the first step, the aqueous tobacco leaf extract was fractionated using semi-preparative liquid chromatography (prep-LC). In the next step, the LC fractions were recombined in an omission approach. In this approach, a series of "pooled" extracts was obtained, and in each extract an LC fraction was omitted. In that way, certain tobacco leaf constituents are eliminated from the "pooled" extracts. All extracts were then concentrated and analyzed by Py-GC–MS using a resistive heating pyrolysis system installed on a thermal desorption unit. The obtained pyrograms were statistically evaluated and compared with a whole extract (all LC-fractions present) using a multivariate chemometric approach and the key LC-fraction was identified that contribute mostly to the formation of typical cigarette smoke aroma. Chemical information from the samples was obtained by analysis of both volatile and non-volatile compounds, followed by processing of chromatograms and reduction in the number of variables using specialized software. Chemometric analysis can rapidly classify and differentiate significant fractions for further analysis. In addition, GC–MS analysis of derivatized LC-fraction was performed to identify the precursors of these aroma compounds and the main constituents were identified as aspartic acid, proline, malic acid and sugars (fructose, glucose and sucrose). These are natural constituents in tobacco leaves and their degradations through Maillard-type reactions contribute significantly to the aroma character of cigarette smoke. The analytical approach was suitable to correlate the effect of different classes of compounds in tobacco leaf to the typical aroma compounds in cigarette smoke. The LC-fraction has the greatest effect on the composition of the volatile fraction of the pyrolysate of an aqueous extract of tobacco leaf. This evidence confirms that the sugars and amino acids detected in that fraction play a major role in the aroma of cigarette smoke. A huge number of amino acids and sugars through Maillard reactions contribute to tobacco leaf and cigarette smoke aroma. Therefore, the described analytical pyrolysis systems will provide an advanced technique for understanding these Maillard reaction pathways. [1]

For more in-depth study, then, analytical method was modified to analyze intermediate compounds in chapter 3. Typically, pyrolysis products are analyzed using Py-GC–MS. However, some products, and especially intermediate compounds, are too polar and thermally unstable to be analyzed directly using GC–MS. Therefore, various derivatization methods are applied in combination with Py-GC–MS. On the other hand, LC–MS can be also applied to analyze polar, non-volatile fractions of pyrolysates, but the combination of pyrolysis with LC–MS is much less documented compared with Py-GC–MS.

In this study, a recently developed resistive heating pyrolysis devices, namely a generic pyrolysis unit (PyroVial, Research Institute for Chromatography, Kortrijk, Belgium) was applied. The PyroVial system is an off-line pyrolysis system based on a modified 2 mL autosampler vial. In this system, the sample is placed in a bottom-tapered quartz insert vial that is inserted in a heating filament, mounted inside the 2 mL vial. After pyrolysis, several options are possible: the headspace gas can be injected in GC, solvent can be added and the dissolved fraction can be analysed by GC or LC and/or derivatization reagent can be added for post-pyrolysis derivatization of the pyrolysate. Firstly, a mixture of glucose and proline was pyrolysed as model. These two compounds were selected because they represent, respectively, an abundant sugar and amino acid content present in tobacco leaf. The volatile fractions of the pyrolysates were analyzed using headspace-GC–MS, semi-volatile using liquid injection GC–MS and non-volatiles using LC–MS. In addition, a tobacco leaf extract was analyzed by Py-LC–MS, whereby the micro-vial pyrolysis simulates the tobacco leaf combustion process. Using MS deconvolution, molecular feature extraction and differential analysis it was possible to identify Amadori

intermediates of the Maillard reaction in the tobacco leaf extract. The intermediate disappeared was the case for 1-deoxy-1-prolino-β-D-fructose or the concentration decreased in the pyrolysate compared to the original extract such as for the 1-deoxy-1-[2-(3-pyridyl)-1-pyrrolidinyl]-β-D-fructose isomers, indicating that Amadori intermediates are important precursors for aroma compound formation. 1-deoxy-1-[2-(3-pyridyl)-1-pyrrolidinyl]-β-D-fructose isomers were identified by LC–Q-TOF-MS analysis in MS/MS mode to determine the possible chemical structures, and using Molecular Structure Correlator (MSC) software. The identified compounds correspond to Amadori rearrangement intermediates, previously reported and naturally present in tobacco leaf and the two isomers were the fructopyranose (major) and fructofuranose (minor) isomers, respectively. Pyrolysis at 500°C led to the formation of pleasant and sweet aromas. 1-deoxy-1-[2-(3-pyridyl)-1-pyrrolidinyl]-β-D-fructose is the Amadori product from glucose and nornicotine, a secondary alkaloid present in *Nicotiana tobacum* L. The novel vial-based resistive heating pyrolysis device, PyroVial, was successfully applied to study Maillard reactions and to identify Amadori intermediates. The obtained information is very useful to further elucidate the formation of aroma compounds during thermal treatment or pyrolysis of natural products. [2]

Finally, advanced comprehensive analytical systems applied to detect a trace level of sulfuric aroma compounds in cigarette smoke in chapter 4. Volatile sulfur compounds in food and beverage have received special attentions due to their extremely low odor threshold levels and high sensory impact, but these compounds are mostly often present at very low levels in complex matrices. Sulfur compounds which are derived from Maillard reaction of sugar and amino acids degradation products also play an important role in the aroma of cigarette smoke. However, the number of identified sulfur compounds in cigarette smoke is still limited due to one of the most complex sample matrices. To analyze volatile sulfur compounds in cigarette smoke, it is therefore essential to have advanced GC separation technique and instrumentation. An effective way to improve the chromatographic resolution and identification capability is through multi-dimensional GC coupled to a hybrid quadrupole time-of-flight mass spectrometer (MDGC–Q-TOF-MS) with element-specific detection.

In this study, a large volume injection (LVI), 10 µL of cigarette smoke extract, followed by selectable ^{1}D/^{2}D GC–Q-TOF-MS that is possible to select one-dimensional (^{1}D) GC separation or heart-cutting two-dimensional (^{2}D) GC separation without any additional instrumental set-up, with parallel sulfur chemiluminescence detection (SCD) was applied for identification of trace sulfur compounds in cigarette smoke. The selectable ^{1}D/^{2}D GC–SCD/Q-TOF-MS system has also the capability to integrate parallel olfactometry in both ^{1}D and ^{2}D separations. To unravel the complexity and identify important sulfur compounds, 28 sulfur fractions selected from ^{1}D GC–SCD on a non-polar pre-column are sequentially transferred onto a polar main-column and then further separated, detected and identified using ^{2}D GC–SCD/Q-TOF-MS. In order to identify each individual sulfur compound, sequential heart-cuts of 28 sulfur fractions from ^{1}D GC to ^{2}D GC were performed with the three MS detection modes (SCD/EI-TOF-MS, SCD/PCI-TOF-MS, and SCD/PCI-Q-TOF-MS). Thirty sulfur compounds were positively identified by MS library search, ^{1}D/^{2}D linear retention indices (LRI), elemental information (sulfur), molecular mass determination using positive chemical ionization (PCI) accurate mass spectra, formula calculation using electron ionization (EI) and PCI accurate mass spectra, and structure elucidation using collision activated dissociation (CAD) of the protonated molecule. Additionally, the best candidate molecular formulas could be obtained for 11 unknown sulfur compounds. Also, sulfur compounds are quantified with the use of the linear and equimolar response of the ^{2}D GC–SCD to sulfur compounds. The determined values of the identified and unknown sulfur compounds were in the range of 10–740 ng mg total particulate matter (TPM)$^{-1}$ (RSD: 1.2–12%, $n = 3$). Forty-one sulfur compounds could thus be determined at ng mg TPM^{-1} levels. Of these sulfur compounds, 13 compounds have not previously been reported in cigarette smoke. [3]

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Through the efforts of this dissertation, innovative analytical work flows designed to lead to an increased understanding of the chemistry of the Maillard reaction and caramelization using real-life samples could be demonstrated. In tobacco science, but also in other fields such as food technology and biological application, sophisticated GC–MS and LC–MS techniques combined with computation analyses for aroma precursors screening, intermediates monitoring, trace level of aroma compounds detection generated by thermal processing and elucidating their formation pathways that has been developed in this dissertation are widely applicable, important and required for advanced research.

[1] Kazuhisa Mitsui, Frank David, Emmie Dumont, Nobuo Ochiai, Hirotoshi Tamura, Pat Sandra. LC fractionation followed by pyrolysis GC–MS for the in-depth study of aroma compounds formed during tobacco combustion. *Journal of Analytical and Applied Pyrolysis*, 116:68–74. (2015)

[2] Kazuhisa Mitsui, Frank David, Bart Tienpont, Koen Sandra, Nobuo Ochiai, Hirotoshi Tamura, Pat Sandra. Analysis of the reaction products from micro-vial pyrolysis of the mixture glucose/proline and of a tobacco leaf extract: Search for Amadori intermediates. *Journal of Chromatography A*, 1422:27–33. (2015)

[3] Nobuo Ochiai, Kazuhisa Mitsui, Kikuo Sasamoto, Yuta Yoshimura, Frank David, Pat Sandra. Multidimensional gas chromatography in combination with accurate mass, tandem mass spectrometry, and element-specific detection for identification of sulfur compounds in tobacco smoke. *Journal of Chromatography A*, 1358:240–251. (2014)

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