NMR based studies on odorant melanoidin interactions in coffee beverages

MICHAEL GIGL, Oliver Frank and Thomas Hofmann

Chair of Food Chemistry and Molecular Sensory Science, Technical University of Munich, michael.gigl@tum.de

Abstract

Freshly brewed coffee is appreciated by consumers all over the world because of its stimulating effect, its characteristic taste centring on sourness and pleasant bitterness and its alluring aroma with characteristic "roasty/sulphurous" odour notes, culminating in the unique flavour sensation of coffee. The molecules responsible for the olfactory sensation of roasted coffee beans and percolated coffee beverages, analysed by means of the molecular sensory science approach are well understood. With previous studies providing qualitative and quantitative data, using aroma extract dilution analysis (AEDA), gas chromatography-olfactometry (GC-O) and headspace GC-MS techniques resulting in comprehensive aroma recombinates consisting of not more than 30 odorants [1-3]. Although the aroma of coffee can be reconstituted rather well, the impact of the melanoidin containing high molecular weight fractions (HMW) on the sensory quality of coffee beverages, is still mostly unclear on a molecular basis. Whereas former studies clearly indicated that especially odour active thiols exhibit high binding affinity to high molecular weight melanoidin fractions of coffee, only covalent interactions have been considered so far. The impact of non-covalent π-π interactions on coffee flavour is still completely unclear [4, 5]. To get detailed insight into the molecular phenomenon of odorant polymer interactions and the sensory impact on coffee flavour perception, a quantitative ^{1}H-NMR based screening approach was developed, which allowed the direct and non-invasive analysis of molecular interactions between key coffee odorants, like 2-furfurylthiol and high molecular weight melanoidin polymers (>10 kDa). A clear distinction between covalent and non-covalent interactions was achieved by monitoring time dependency of odorant polymer interactions, with 2-furfurylthiol exhibiting π-π interactions as well as covalent bindings. In contrast, pyrazines and hydroxyphenols showed only non-covalent π-π stacking, whereas aldehydes incubated with HMW material showed only covalent interactions at prolonged incubation times. Furanones, as well as diketones showed no interactions with the HMW. Human sensory experiments with isolated HMW material >10 kDa and a full aroma recombinate of coffee were well in the line with the findings from the NMR based approach. A drastic reduction of "roasty/sulphurous" aroma notes in combination with a decrease in overall coffee-like odour quality, as well as an increased "sweetish/caramel-like" flavour was perceivable upon incubation of coffee melanoidins with the aroma recombinate. The lack of binding affinity of the sweetish/caramel smelling 4-hydroxy-2,5-dimethyl-3(2H)-furanone in combination with the high binding affinity of coffee thiols provides explanation of the sensory evaluation and might be the reason for the fast disappearance of the "roasty/sulphurous" aroma impressions of a freshly prepared coffee brew.

Keywords: NMR, coffee aroma staling, sensory experiments, covalent interactions, π-π interactions

Introduction

Unfortunately, the aroma of a freshly prepared coffee beverage is very unstable and deteriorates within minutes after the preparation of the brew [5]. Molecular reactions taking place during storage of coffee beverages were analysed in previous studies with the aim of clarifying the reasons for the unstable nature of the coffee aroma on a molecular basis. A drastic influence of storage on the concentrations of the "roasty/sulphurous" smelling thiols 3-methyl-2-butene-1-thiol, 3-mercapto-3-methylbutyl formate, 2-methyl-3-furanthiol, methanethiol, and, the major key aroma compound of roasted coffee 2-furfurylthiol (FFT) could be shown in the literature after keeping a freshly brewed coffee warm in a thermos flask. A biomimetic in-bean model roasting approach clearly demonstrated the high thiol binding affinity of low molecular weight (LMW) compounds derived from chlorogenic acid degradation [6]. Consequently, the conjugation of FFT with di- and trihydroxybenzenes was confirmed using LC-MS and NMR techniques [7, 8]. Furthermore, furan derivatives, like furfurylalcohol, were shown to interact with dihydroxy- and trihydroxybenzenes and lead to the detection of numerous (furan-2-yl)methylated benzene diols and triols in brewed coffee [9]. Recently, an untargeted UPLC-TOF-MS screening approach in combination with statistical S-plot analysis was used to identify reaction products formed between Strecker aldehydes, present in the coffee aroma and chlorogenic acid, quinic acid and a quinic acid lactone [10].

Additionally, besides adducts formed between LMW coffee compounds and odorants during roasting and/or storage of the percolated brew, the effect of HMW coffee melanoidins was evaluated regarding their influence on the aroma staling phenomenon [4, 5]. These brown coloured macromolecules are generated by thermal processing of foods, like the roasting of coffee and embody a very heterogeneous compound class [11–15].

These melanoidin populations contain a plethora of reactive compounds and/or side-groups (i.e. phenols), and were shown to exhibit high binding affinities to odour active thiols, like 2-fufurylthiol by means of gas chromatography(GC) -mass spectrometry (MS) and human sensory experiments [5]. Using coffee-like model

DOI:10.5281/zenodo.5705478

systems, it could be demonstrated, that odour-active thiols can react with pyrazinium di-cations, the oxidation products of the "CROSSPY" radicals. These radical species were reported to be key intermediates in melanoidin formation [4, 16, 17].

However, the discovery of molecular interaction between volatile and non-volatile coffee constituents was restricted mainly to the occurrence of covalent reactions as a result of the analytical methods that were used. Interactions such as complex formation by e.g. non-covalent (π-π), dipole-dipole or van der Waals interactions were revealed to be disbanded during liquid chromatography because of the dynamic equilibrium between lightly associated complexation partners [18]. Thus, bound and unbound states of the ligands could not be differentiated, and state of the art GC or UHPLC analysis remains not suitable for observing dynamic non-covalent interactions. To overcome these challenges, NMR-based methodologies were developed to enable the investigation of binding affinities of selected LMW ligands, by monitoring their specific resonance signals with regards to signal shape, multiplicity, and chemical shift.

In the literature non-covalent interactions were reported to influence proteins and ligands [19-22], the oral astringency of polyphenols [18], as well as the co-pigmentation of anthocyanins [23].

Specifically, for coffee, a π-π complex encompassing chlorogenic acid and caffeine is known from literature, signifying the possibility of π-π stacking to occur in percolated coffee [24-26]. In order to study the influence of non-covalent interactions on aroma active compounds, an NMR based approach was shown to yield promising results for red wine aroma perception. It was reported, that the 2-methylpyrazine and vanillin showed a high binding affinity to red wine polyphenols like gallic acid and naringin [27]. So far, non-covalent interactions were not discussed in the context of olfactory perception of coffee brew and the loss of the characteristic "roasty/sulphurous" aroma impressions during storage of coffee beverages. Thus, the object of the current study is to obtain comprehensive and detailed understanding of the interaction between key coffee odorants and the melanoidin containing fraction of coffee, as well as to depict the sensory impact on coffee aroma perception.

First, human sensory experiments were performed, in order to assess the effects of melanoidin addition on the aroma profile of coffee, followed by a qualitative and quantitative ^{1}H-NMR based approach to further evaluate and classify these interactions on a molecular basis.

Experimental

Preparation of coffee beverages

A single-dose capsule machine (De`Longhi Nespresso Inissa EN 80.B) was used to percolate commercially available coffee capsules. The coffee Capsa Lungo Mild Roast (Dallmayr Capsa, Munich, Germany) was brewed with water (104 mL water/capsule), resulting in standard coffee beverages with concentrations of 5.4 g/100 mL. After rapidly cooling to room temperature in an ice bath, five freshly prepared standard coffee beverages were pooled (n=5, 520 mL) and submitted to the ultrafiltration process.

Isolation and purification of HMW material from coffee by means of ultrafiltration

Isolation of high molecular weight (HMW) material from coffee was achieved with a crossflow ultrafiltration system (Sartorius Stedim Biotech, Göttingen, Germany) and a molecular weight cut-off filter (10 kDa, Sartorius Stedim Biotech, Göttingen, Germany) resulting in a HMW fraction >10 kDa, which was used for the present studies. The HMW fraction was purified by washing with water (5 L) and the cleansing progression was checked by acquiring ^{1}H-NMR spectra after each flushing period (1 L).

Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H-NMR measurements were performed on a Bruker AVANCE III 500 MHz system equipped with a cryo-TCI Probe at 300 K in 5 mm × 7" NMR tubes (Z107374 USC tubes, Bruker, Faellanden, Switzerland). Data handling was done using the Topspin software version 3.1 and 4.0.

Quantitative NMR spectroscopy (qHNMR) was achieved with the ERETIC 2 procedure, based on the PULCON methodology, according to literature [28]. Spectrometer calibration was done via L-tyrosine (5.21 mmol/L) as the external reference, integrating the specific proton resonance signal at 7.10 ppm (m, 2H).

Reference compounds were weighed in volumetric flasks and filled with water, giving concentrations of 2-25 mmol/L. Before NMR measurements, solutions of reference compounds and isolated HMW fractions (540 µL) were spiked with a NMR buffer (60 µL, pH 5.5). The buffer contained potassium dihydrogen phosphate (10.2 g), potassium hydroxide (1.5 g), trimethylsilyl propionic acid (50 mg) and sodium azide (5 mg) dissolved in D_2O (40 mL). Then, a deuterium chloride solution (4.0 mol/L in D_2O) was used to adjust the pH value to 5.5 and made up to 50 mL with D_2O.

Sensory experiments

Human sensory experiments were carried our as described in the literature [1]. Samples (20 mL) were prepared in phosphate buffered water (0.1 mol/L, pH 5.5) and transferred to glass beakers (diameter 45 mm, capacity 45 mL). Odourless sugar colour was used to negate to optical differences in samples in combination with red lighting in the sensory booths. Sensory analyses were carried out in a sensory panel room with individual cabins at 22-25 °C.

Panellists (8 females, 7 males; 23−32 years in age) were requested to assess the intensities of the aroma attributes "sweetish/caramel-like", "earthy", "roasty/sulphurous" and "smoky", "seasoning-like" and "fruity" on a scale from 0 (absent) to 3 (very strong). Additionally, the following reference compounds were presented to the panellists: 4-hydroxy-2,5-dimethyl-3(2H)-furanone for "sweetish/caramel-like", 2,3-diethyl-5-methoxypyrazine for "earthy", a mixture of 2-furfurylthiol, 3-mercapto-3-methylbutanol and 3-mercapto-3-methylbutyl formate representing "roasty/sulphurous", 2-methoxyphenol for "smoky", 3-hydroxy-4,5-dimethylfuran-2(5H)-one for "seasoning-like" and a mixture of ß-damascenone and acetaldehyde for "fruity".

Results and discussion

First, the high molecular fraction (HMW) of a coffee beverage was isolated via a crossflow ultrafiltration system using a cut-off membrane (10 kDa). In order to verify the purification of the HMW and to ensure that all small molecules were flushed out, ^{1}H NMR spectra were recorded after each washing cycle with water (1 L). Aliquots of the isolated and purified HMW fraction >10 kDa were used for human sensory experiments and NMR studies.

To evaluate the role of HMW coffee melanoidins on coffee aroma perception, first, a basic aroma recombinate was prepared consisting of 25 aroma active compounds displayed in Figure 1 [1]. With this recombinate, aroma profiles of the aqueous coffee aroma recombinate, and the aroma recombinate with the HMW fraction >10 kDa added in original concentration were determined. All samples were incubated for 60 min at room temperature, to allow possible interactions between odorants and high molecular weight coffee melanoidins.

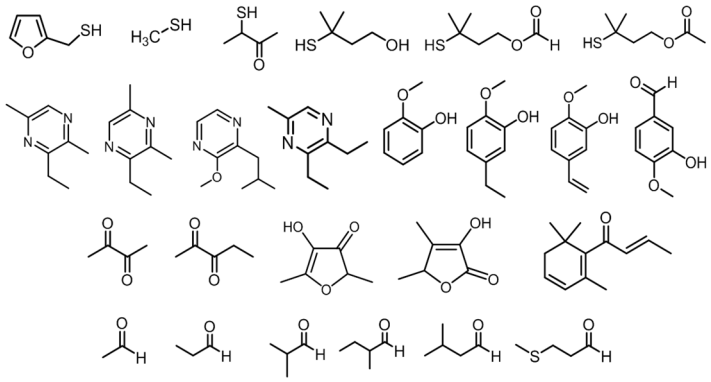

Figure 1*: Structures of odorants present in the coffee aroma recombinate.*

The resulting aroma profiles showed a major influence of added melanoidins to the aqueous aroma recombinate. The attributes "roasty/sulphurous", "earthy", and "smoky" were reported to be less prominent in the samples containing added melanoidins (Figure 2). The intensity of "roasty/sulphurous" aroma notes of the samples with added HMW fraction were rated with 1.6 and were substantially reduced, compared to the recombinate without added melanoidins (rated 2.2). A similar trend could be shown for the aroma attributes earthy and smoky. On the other hand, the aroma intensity of "sweetish/caramel-like" aroma notes was rated with 1.9 in the samples with added HMW fraction and was therefore perceived stronger compared to the samples without HMW, which were rated with just 1.5 by the panellists. These findings are well in line with the literature, where storage time [3] and melanoidin addition severely influenced the rated aroma intensities of coffee aroma attributes [4, 5].

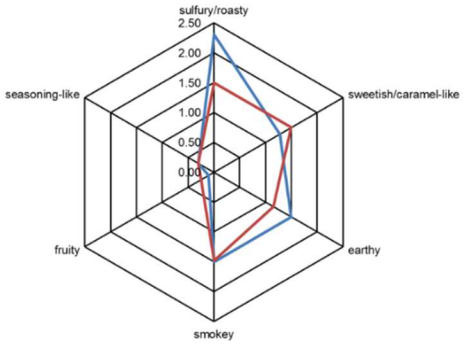

Figure 2*: Aroma profiles of the aqueous aroma recombinate (blue) and the aroma recombinate with added melanoidins (red) in original coffee concentration.*

In order to elucidate the molecular mechanisms involved in the aroma staling phenomenon, observed between the HMW fraction of coffee and key odour compounds an ^{1}H-NMR based approach was used. NMR spectroscopy is a suitable method to investigate non-covalent interactions on a molecular level [19]. The NMR based method principally makes use of ^{1}H-NMR signal attenuation as well as chemical shift difference of low molecular weight ligands due to interactions with HMW compounds. The resonance signal of interacting ligands shows reduced intensity and consequently a smaller integral. Comparing the integral of samples with HMW material to aqueous isomolar references without added HMW fraction enables the evaluation binding ratios of the respective odorants.

Consequently, the odorants 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 2,3-buranedione, 3-methylbutanal, 3-mercapto-3-methylbutyl formate, 2-furfurylthiol, 2,3-diethyl-5-methylpyrazine, 2-methoxyphenol and (E)-ß-damascenone as representatives of each compound class (furanones, diketons, aldehydes, non-aromatic thiols, aromatic thiols, pyrazines, methoxyphenols, and terpenes) were used for the NMR studies. Solutions of the respective aroma compounds were prepared, and the exact concentration was quantified in a buffered system representing coffee conditions via qHNMR. Samples for NMR analyses were prepared by adding odorants (5 mmol/L) to the HMW fraction (>10 kDa) in native coffee concentration. As a reference, all odorants were prepared without HMW addition. After incubating the samples for 60 min at room temperature quantitative ^{1}H-NMR spectra were acquired. To avoid chemical shift differences caused by the pH value, all solutions were buffered at pH 5.5. The NMR spectra of odorants with the added HMW fraction were evaluated in comparison to the aqueous reference.

NMR based screening clearly showed the influence of melanoidin addition on aroma active compounds. For example, the representative substances for the odorant classes pyrazines (2,3-diethyl-5-methylpyrazine), and aromatic thiols (FFT) were highly influenced by addition of the HMW fraction. The signal shape as well as the signal width, measured as the full width at half maximum (FWHM), indicated high binding affinity to macromolecular coffee melanoidins (Figure 3). The FWHM of 2,3-diethyl-5-methylpyrazine changed from 2.02 Hz in the aqueous reference to 4.16 Hz in the sample with added melanoidins and therefore led to significant signal broadening of 2.14 Hz and was shifted to higher frequencies by 1.42 Hz, which is supported by literature, where it is reported that compounds exhibiting non-covalent π-π interactions studied by NMR spectroscopy often show line broadening and shift of resonances to higher frequencies [36, 37, 18]. In comparison, the aroma active compounds 4-hydroxy-2,5-dimethyl-3(2H)-furanone and 2,3-butanedione, representing furanones and diketones are mostly unaffected by the addition of the HMW fraction >10 kDa. The resonance signal of 4-hydroxy-2,5-dimethyl-3(2H)-furanone showed matching chemical shift, signal shape and FWHM compared to the aqueous reference, well in the line with the observations from the sensory analysis, that no interaction with coffee melanoidins takes place. The aroma active compounds 3-methylbutanal and 3-mercapto-3-methylbutylformate were analysed exemplary for aldehydes and non-aromatic thiols. Evaluation of the signal shape of the methyl protons of 3-methylbutanal showed reduced signal intensity after 60 min of incubation with the HMW fraction of coffee, suggesting potential interactions with melanoidins.

The coffee melanoidins revealed a drastic influence on pyrazine, methoxyphenol, terpene and aromatic thiol. While the aldehyde and non-aromatic thiol were only slightly influenced by the addition of melanoidins, furanones and diketones were unaffected by the presence of HMW material.

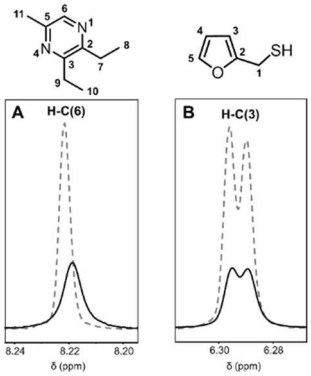

Figure 3*: Excerpts of qHNMR spectra (500 MHz; H_2O/D_2O, 9/1, v/v, pH 5.5; 300 K) of the two key coffee odorants 2,3-diethyl-5-methylpyrazine (**A**) and 2-furfurylthiol (**B**) with (black line) and without (dotted grey line) added melanoidin fraction >10 kDa in original coffee concentration.*

Since these model studies with selected odorants and melanoidins were able to confirm the results of the human sensory experiments, a coffee brew in its entirety was analysed regarding the occurrence of aroma binding interaction. Therefore, a coffee beverage was freshly brewed, cooled to room temperature and an aliquot spiked directly with 2,3-diethyl-5-methylpyrazine. The mixture was incubated for 60 min at room temperature and subjected to NMR measurement. In comparison to an aqueous reference solution without coffee, the pyrazine resonance signal of H-C(6) displayed significant line broadening, reduced intensity and a shift to higher frequencies, unequivocally demonstrating a reduction of free pyrazine upon the incubation with coffee brew from 47.8 mmol/L (reference solution) to 24.4 mmol/L resulting in a recovery rate of 51%, revealing the same interaction behaviour as demonstrated in the model experiments shown above. Consequently, the non-covalent interactions could also be observed in a real coffee beverage, validating the suitability of the NMR based approach to determine binding activities of key coffee odorants to the macromolecular melanoidins.

Conclusion

In summary the quantitative qHNMR based approach allowed the evaluation of binding affinities and the characterization of interactions between LMW and HMW coffee constituents. Results from the qHNMR experiments revealed structure activity relationships between the chemical structure of the odour active compounds and their interaction activity. In addition, the conclusions drawn from model systems were effectively confirmed to occur in freshly prepared coffee beverages. In addition, the human sensory experiments were in good agreement with the NMR data and provided explanation of the described interactions. The overall aroma of coffee was shifted to less "roasty" and more "sweet/caramel-like" aroma notes, by selectively interacting with "roasty" smelling thiols, like FFT as well as the absence of interactions with "caramel-like" odour compounds, i.e. 4-hydroxy-2,5-dimethyl-3(2H)-furanone.

References

1. Mayer F, Czerny M, Grosch W. Sensory study of the character impact aroma compounds of a coffee beverage. Eur Food Res Technol. 2000;211(4):272–276.
2. Semmelroch P, Grosch W. Analysis of roasted coffee powders and brews by gas chromatography-olfactometry of headspace samples. LWT-Food Sci Technol. 1995;28(3):310–313.
3. Semmelroch P, Grosch W. Studies on Character Impact Odorants of Coffee Brews. J Agric Food Chem. 1996;44(2):537–543.
4. Hofmann T, Schieberle P. Chemical Interactions between Odor-Active Thiols and Melanoidins Involved in the Aroma Staling of Coffee Beverages. J Agric Food Chem. 2002;50(2):319–326.
5. Hofmann T, Czerny M, Calligaris S, Schieberle P. Model Studies on the Influence of Coffee Melanoidins on Flavor Volatiles of Coffee Beverages. J Agric Food Chem. 2001;49(5):2382–2386.
6. Müller C, Hofmann T. Screening of raw coffee for thiol binding site precursors using "in bean" model roasting experiments. J Agric Food Chem. 2005;53(7):2623–2629.
7. Müller C, Hofmann T. Quantitative studies on the formation of phenol/2-furfurylthiol conjugates in coffee beverages toward the understanding of the molecular mechanisms of coffee aroma staling. J Agric Food Chem. 2007;55(10):4095–4102.
8. Müller C, Hemmersbach S, Slot G, Hofmann T. Synthesis and structure determination of covalent conjugates formed from the sulfury-roasty-smelling 2-furfurylthiol and di- or trihydroxybenzenes and their identification in coffee brew. J Agric Food Chem. 2006;54(26):10076–10085.
9. Kreppenhofer S, Frank O, Hofmann T. Identification of (furan-2-yl)methylated benzene diols and triols as a novel class of bitter compounds in roasted coffee. Food Chem. 2011;126(2):441–449.

10. Gigl M, Frank O, Barz J, Gabler A, Hegmanns C, Hofmann T. Identification and Quantitation of Reaction Products from Quinic Acid, Quinic Acid Lactone, and Chlorogenic Acid with Strecker Aldehydes in Roasted Coffee. J Agric Food Chem. 2021;69(3):1027–1038.
11. Nunes F, Coimbra M. Chemical Characterization of the High-Molecular-Weight Material Extracted with Hot Water from Green and Roasted Robusta Coffees As Affected by the Degree of Roast. J Agric Food Chem. 2002;50(24):7046–7052.
12. Moreira A, Nunes F, Domingues M, Coimbra M. Coffee melanoidins. Structures, mechanisms of formation and potential health impacts. Food Funct. 2012;3(9):903–915.
13. Bekedam E, Schols H, van Boekel M, Smit G. Incorporation of chlorogenic acids in coffee brew melanoidins. J Agric Food Chem. 2008;56(6):2055–2063.
14. Bekedam E, Loots M, Schols H, van Boekel M, Smit G. Roasting effects on formation mechanisms of coffee brew melanoidins. J Agric Food Chem. 2008;56(16):7138–7145.
15. Bekedam E, Schols H, van Boekel M, Smit G. High molecular weight melanoidins from coffee brew. J Agric Food Chem. 2006;54(20):7658–7666.
16. Hofmann T; Bors W; Stettmaier K.: CROSSPY. A Radical Intermediate of Melanoidin Formation in Roasted Coffee. Free Radicals in Food. 2002: 49–68.
17. Hofmann T, Bors W, Stettmaier K. Radical-assisted melanoidin formation during thermal processing of foods as well as under physiological conditions. J Agric Food Chem. 1999;47(2):391–396.
18. Delius J, Frank O, Hofmann T. Label-free quantitative 1H NMR spectroscopy to study low-affinity ligand-protein interactions in solution. A contribution to the mechanism of polyphenol-mediated astringency. PloS One. 2017;12(9):e0184487.
19. Burley S K, Petsko G A, Aromatic-aromatic interaction. A mechanism of protein structure stabilization. Science (New York, N.Y.). 1985;4708(229):23–28.
20. Wang Z-X, Ravi Kumar N, Srivastava D K. A novel spectroscopic titration method for determining the dissociation constant and stoichiometry of protein-ligand complex. Anal Biochem. 1992;206(2):376–381.
21. Marsili S, Chelli R, Schettino V, Procacci P. Thermodynamics of stacking interactions in proteins. Phys Chem Chem Phys. 2008;10(19):2673–2685.
22. Wilson K A, Kellie J L, Wetmore S D. DNA-protein π-interactions in nature. Abundance, structure, composition and strength of contacts between aromatic amino acids and DNA nucleobases or deoxyribose sugar. Nucleic Acids Res. 2014;42(10):6726–6741.
23. Mistry T V, Cai Y, Lilley T H, Haslam E. Polyphenol interactions. Part 5. Anthocyanin co-pigmentation. J Chem Soc. 1991;2(8):1287-1295.
24. Sondheimer E, Covitz F, Marquisee M J. Association of Naturally Occuring Compounds, the Chlorogenic Acid-Caffeine Complex. Arch Biochem Biophys. 1961;93:63–73.
25. Horman I, Viani R. The Nature and Conformation of the Caffeine-Chlorogenate Complex of Coffee. J Food Sci. 1972;37(6):925–927.
26. Martin R, Lilley T H, Falshaw C P, Haslam E, Begley M J, Magnolato D. The Caffeine-Potassium Chlorogenate Molecular Complex. Phytochemistry. 1987;26:273–279.
27. Jung D M, de Ropp J S, Ebeler S E. Study of Interactions between Food Phenolics and Aromatic Flavors Using One- and Two-Dimensional 1H NMR Spectroscopy. J Agric Food Chem. 2000;48(2):407−412.
28. Frank O; Kreissl J; Daschner A; Hofmann T.: Accurate determination of reference materials and natural isolates by means of quantitative (1)H NMR spectroscopy. J Agric Food Chem. 2014;62(12):2506–2515.