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# Fully Automated Identification of Coffee Species and Simultaneous Quantification of Furfuryl Alcohol Using NMR Spectroscopy

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## Abstract

**Background:** Coffee is a popular beverage with two species, *Coffea canephora* and *C. arabica*, being commercially exploited. The quality and commercial value of coffee is dependent on species and processing. *C. arabica* typically obtains a higher price on the market compared to *C. canephora*. Coffee beans undergo roasting during processing, resulting in the formation of flavor compounds including furfuryl alcohol which has been classified by the International Agency for Research on Cancer as possibly carcinogenic to humans (Group 2B).
**Objective:** The aim of this study was to identify coffee species and other properties using nuclear magnetic resonance (NMR) spectroscopy, specifically to conduct quantification of the roasting process contaminant furfuryl alcohol.
**Method:** The quantification of furfuryl alcohol was performed from the NMR spectra using the pulse length-based concentration (PULCON) methodology. Prior to NMR analysis, samples were extracted using deuterated chloroform.
**Results:** Roasting experiments identified the maximum roasting temperature to be the most significant factor in the formation of furfuryl alcohol. Among the coffee species, *C. canephora* was found to contain a relatively lower amount of furfuryl alcohol compared to *C. arabica*. The roasting of wet processed coffee resulted in higher contents of furfuryl alcohol. Geographical origin and variety within species had no influence on the furfuryl alcohol content.
**Conclusion:** Validation results show that NMR spectroscopy is fit-for-purpose to obtain targeted information of coffee samples.
**Highlights:** The PULCON NMR methodology allows a simple, rapid and accurate determination of constituents of coffee.

Of the more than 70 known *Coffea* species only *Coffea arabica* and *C. canephora* are of great economic importance contributing to approximately more than 99% of global production. *C. canephora*, commonly known as "Robusta", accounts for approximately 39% of the production whereas *C. arabica* accounts for 61% of the 9500 metric tons sold globally in 2017 (1). Other coffee species of some economic interest are *C. liberica* and *C. racemosa* (2, 3).

Ecologically, *C. canephora* is grown in low altitude regions whereas *C. arabica* thrives better on mountain slopes. Generally,

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the cultivation of *C. arabica* is more complex and expensive compared to *C. canephora*. *C. arabica* coffee is preferred by a majority of consumers compared to *C. canephora* by virtue of its rich aroma and flavor (4, 5).

The popular and well-known stimulant effect of coffee is attributed to the alkaloidal constituent caffeine, which originally acts as a natural defense against browsing herbivores, insects, fungi, bacteria, and mollusks such as snails (6). The amount of caffeine in coffee beans is mainly dependent on the mother plants' type and variety. The processing of coffee beans has been shown not to affect the caffeine content. The seeds of *C. canephora* generally contain higher amounts of caffeine than those of *C. arabica* (3).

The lipid fraction of the coffee bean comprises of about 15% diterpenes and their esters. The major diterpenes found in coffee include cafestol and 16-O-methylcafestol (OMC) (7). Cafestol is found in both *C. canephora* and *C. arabica* whereas OMC is specific only to *C. canephora* (8, 9). Therefore, it is feasible to distinguish *C. arabica* and *C. canephora* coffees from each other and detect food fraud by substitution of *C. arabica* with the cheaper *C. canephora* beans using OMC as a marker. Additionally, another diterpene, kahweol, which is found in both types of coffee, but in significantly higher amounts in *C. arabica* coffee may be used as an authenticity marker for coffee (2, 4).

After harvesting, coffee cherries are subjected to cleaning and careful elimination of defective or immature fruit. These initial processes impact on the final quality of the roasted coffee. After cleaning and sorting the cherries, it is necessary to remove the pulp of the coffee cherries either by dry, semi-washed or wet processing. In dry processing, the coffee cherries are spread out and sun-dried for 3-9 days followed by removal of the dried pulp together with parchment skin by a peeling machine. The silver-colored skin may also be removed. Dry processing is preferred for *C. canephora* coffee. In semi-washed processing, the pulp is mechanically separated followed by drying without undergoing fermentation. The other steps are similar to those involved in dry processing (2, 10, 11).

Contrarily, wet processing entails mechanical removal of the coffee cherries' flesh followed by fermentation of the beans for 12-48 h in basins under a controlled water flow. Microorganisms and enzymes from the coffee degrade the residual pulp, making it easy to be carried off by the flowing water. With the aid of a peeling machine, the parchment skin is removed. In order to obtain high-quality coffee, the silver skin may be additionally removed by polishing followed by cleaning with water (2, 10, 11).

The roasting of coffee beans is the essential process affecting the characteristics of the coffee such as color, flavor and taste. Roasting is usually done above 200°C and at different temperature-time profiles (12–14). The color of the roasted coffee is almost entirely due to non-enzymatic browning reactions such as caramelization and the Maillard reaction. Roasting is accompanied by an increase in volume and a mass reduction. The mass reduction is partly due to the evaporation of water and loss of volatile compounds (15). The temperature change throughout the roasting process determines the rate of decomposition of organic materials in coffee with initial changes in the coffee bean starting to appear from 50°C onwards. These changes include protein coagulation and evaporation of water. Non-enzymatic browning reactions and decomposition of organic substances occur at temperatures above 100°C and are marked by smoldering of the coffee. The decomposition of organic substances yields carbon dioxide and carbon monoxide alongside water steam. Steam is formed at 150°C resulting in a significant increase in volume. At temperatures above 180°C, the coffee bean's furrow bursts open giving bluish smoke (16, 17).

Coffee roasting results in numerous flavor profiles with a total of 850 volatile compounds reported in roasted coffee including furfuryl alcohol (2). Furfuryl alcohol, a food processing contaminant, has also been reported in other foods such as cocoa, roasted almonds, bread and honey (18, 19). Furfuryl alcohol may be formed either from quinic acid or caffeic acid (20, 21). Quinic acid is a natural constituent of green coffee whereas caffeic acid is released only during roasting from chlorogenic acid. Both organic acids may be converted by several successive dehydrations and radical reactions into furfuryl alcohol (22, 23). Despite the International Agency for Research on Cancer's classification of furfuryl alcohol as a possible carcinogen (Group 2B) (24), there is a scarcity of data regarding its content in coffee (18).

Several analytical techniques such as high performance liquid chromatography (25), gas chromatography with flame ionization detection (26), gas chromatography-mass spectrometry (27, 28), proton transfer mass spectrometry (29), nuclear magnetic resonance (NMR) spectroscopy (4), isotope-ratio mass spectrometry (30), near-infrared spectroscopy (31, 32), electronic nose (33), flame atomic absorption spectrometry (34) and attenuated total reflectance Fourier transform infrared spectroscopy (31), among others, have been reported in the literature for quantitative determination of coffee constituents and screening of coffee adulteration. This study characterizes the constituents of coffee using $^{1}$H NMR spectroscopy with special focus on the process contaminant, furfuryl alcohol. The quantification of coffee constituents was performed using pulse length-based concentration (PULCON) determination, which uses an external reference standard, the so-called "Quantref" (quantification reference solution). The influence of the roasting process on the formation of furfuryl alcohol was also investigated.

## Experimental

### Reagents

Deuterated chloroform (99.8%), tetramethylsilane (TMS) (99.9%), O-phosphoric acid (85%), citric acid monohydrate (99.5%) were from Roth (Karlsruhe, Germany) while acetone-$d_6$ was from Eurisotop (Saarbrücken, Germany). Cyclohexane (99.5%), dimethylsulfoxide-$d_6$ with 0.3% tetramethylsilane (99.8%), methanol-$d_4$ (99.8%), 3-(trimethylsilyl)-propionate-$d_4$ (TSP) (98%) were from Merck (Darmstadt, Germany). Deuterium water (99.9%), ethylbenzene (99.8%), furfural (99%), furfuryl alcohol (97.5%), 2-furanmethanethiol (98%), 2-methoxy-4-vinylphenol (98%), sodium-L-lactate (99%), taurine (99%) and 1, 2, 4, 5-tetrachloro-3-nitrobenzene (TCNB) were from Sigma-Aldrich (Taufkirchen, Germany).

### Preparation of NMR Solvent-TMS Mixture, Quantref and Control Solutions

TSP solution was prepared by dissolving 0.5 g of TSP in 50 mL $D_2O$. Samples and reference standards for NMR spectroscopy were dissolved in a mixture 500 mL $CDCl_3$ and 0.5 mL TMS. The Quantref was a mixture comprising of 46.03 mg TCNB and 53.78 mg ethylbenzene dissolved in $CDCl_3$ to 10 mL, and the coffee control solution was prepared by dissolving 25.02 mg TCNB with $CDCl_3$ to 5 mL.

### Coffee Samples

A total of 57 authentic samples of green coffee beans were collected from farms in Brazil and India. The samples from Brazil

comprised of 31 *C. arabica* and one *C. canephora* whereas those from India consisted of 14 *C. canephora* and 11 *C. arabica*. Complete sample information including variety and processing method is provided in Table 1.

For a retrospective analysis, all measured NMR spectra of coffee samples submitted to Chemisches und Veterinäruntersuchungsamt (CVUA)-Karlsruhe in the years 2015-2017 in the context of official controls (n = 582) were re-evaluated for the occurrence of furfuryl alcohol. The retrospective samples were measured and evaluated using our previously described protocol using quantification with TCNB as an internal standard (4).

## Development of an Extraction Method for Furfuryl Alcohol

### *Solvent optimization*

In order to select a suitable solvent for the extraction of furfuryl alcohol and other relevant constituents of coffee such as caffeine and hydroxymethyl furfural (HMF), kahweol and OMC, various deuterated solvents namely $CDCl_3$, acetone-$d_6$, dimethyl sulfoxide-$d_6$ (DMSO-$d_6$), methanol-$d_4$ and $D_2O$ were investigated. Commercial coffee samples were used in the development and optimization of the extraction method.

Additionally the extraction efficiency for mechanical shaking and ultrasonication of samples was investigated.

### *Mechanically shaken samples*

Approximately 200 mg of roasted and ground coffee were suspended in 1.5 mL $CDCl_3$-TMS solution. The mixture was then mechanically shaken for 20 min at 350 rpm. The solution was then centrifuged in tubes with micro-filters (0.45 µm) at 4200 rpm for 20 min. 600 µL of the centrifugate was put into NMR tubes for analysis. Samples of the coffee powder were similarly suspended in either $CDCl_3$ or the other deuterated solvents and mechanically shaken followed by centrifugation. Furthermore before NMR measurement, the mixture was filtered through a syringe filter (0.45 µ). 600 µL of the filtrate was then transferred to an NMR tube for analysis.

### *Ultrasonicated samples*

Roasted and ground coffee (600 mg) was suspended in 4.5 mL $CDCl_3$-TMS solution followed by ultrasonication in a water bath for 20 min. The resultant solution was centrifuged at 4200 rpm for 8 min. The centrifugate was then filtered through a syringe filter (0.45 µ) before 600 µL of the filtrate was placed into NMR tubes for spectroscopic analysis. In order to ensure complete extraction of the analytes, a second extraction was carried out on the sedimented coffee residue. The first supernatant was carefully removed then 2.5 mL of $CDCl_3$-TMS solution was added to the coffee sediment. The slurry was re-extracted in an ultrasonic bath for 20 min and finally centrifuged again at 4200 rpm for 8 min. The mixture was then filtered through a 0.45 µm PTFE-syringe filter. Six hundred microliters of the filtrate was pipetted into an NMR tube for analysis.

## NMR Spectroscopic Equipment and Quantification by the PULCON Method

Three 400 MHz (9.4 T) field strength spectrometers were used: an AVANCE 400 Ultra Shield with a 5 mm PASEI 1 H/D 13 C probe, an Ascend 400 with a PA BBO BB 1 H/D 19 F probe and another Ascend 400 with a PA BBI 400SI probe (each from Bruker, Rheinstetten, Germany). All samples were measured in 5 mm sample tubes (NMR tube Boro 300-5-7) (Deutero, Kastellaun, Germany).

The $^1$H-NMR parameters were as follows: pulse program zg30, temperature 300 K, data points 131 072, pulse 30°, relaxation delay 30 s, acquisition time 7.97 s, dummy scans 2, summated transients 64, spectral width ca. 20.5617 ppm, receiver gain 45.2.

The PULCON NMR method which utilizes an external standard was applied for quantification of the target analytes in coffee. For this purpose, the integrals of the NMR signals of the sample to which an external reference (QuantRef) substance had been added were integrated. The QuantRef sample usually contains one or more substances of known concentrations, dissolved in the same solvent as the sample. With the known concentrations and molar mass of the reference substances (RS), the corresponding integral and measuring parameters, an electronic reference to access in vivo concentrations (ERETIC) factor was determined using the following formula:

$$\mathrm{ERETIC} = \frac{I_{RS} \cdot SW \cdot M_{RS}}{SI \cdot C_{RS} \cdot NH_{RS}} \quad (1)$$

where $I$ is the absolute integral of the selected signal, $SW$ is the spectral width, $M$ is the molecular mass, $SI$ provides information on the number of data points (size of real spectrum), $C$ is the concentration of the reference substance and $NH$ is the number of protons giving rise to the signal. An average of the individual *ERETIC* factors obtained from several NMR signals is used.

For quantification purposes, specific, non-overlapping NMR signals of the analytes in coffee sample were integrated. The concentration, $C_x$, of the substance examined was then calculated as follows:

$$C_x = \frac{I_X \cdot SW \cdot M_X \cdot NS_{RS} \cdot P1_X}{SI \cdot ERETIC \cdot NH_X \cdot NS_X \cdot DF \cdot P1_{RS}} \quad (2)$$

The parameters $SW$ and $SI$ refer in this case to the measurement of the analyte sample. $X$ and $RS$ indicate the analyte or the reference sample, respectively. The molecular mass $M$ refers to the substance that is responsible for the integral $I$ of the signal, as well as the number of protons $NH$. $P1_X$ and $P1_{RS}$ are the pulse lengths and $NS_X$ and $NS_{RS}$ are the summated transients of the different measurements. If necessary, the dilution factor, $DF$, of the sample was also included in the formula.

The reference substances used as QuantRef for the present work were TCNB and ethylbenzene. Another TCNB-solution with a known concentration was used as a control solution, it was measured at the end of each sample series. The stability of the Quantrefs was monitored by NMR measurements daily for 2 weeks. The following integration regions (ppm) were used: OMC 3.125 to 3.185 ($NH = 3$), caffeine 3.38 to 3.44 ($NH = 3$), kahweol 5.85 to 5.925($NH = 1$), furfuryl alcohol 7.39 to 7.411($NH = 1$) and HMF 9.67 to 9.69 ($NH = 1$).

A Matlab script was used for quantitative PULCON evaluation with the formulae specified above. Before quantification, the NMR spectra were preprocessed. The pre-processing methods included baseline and phase correction, signal-picking and integration, conducted using the automation in Bruker TopSpin. Following this pre-processing, the spectra were imported into Matlab.

## Roasting Experiments

Approximately, 50 g of the different green coffee samples were roasted using an electric coffee roaster (Ikawa Pro Coffee Roaster, Ikawa Ltd, London, UK) with a maximum volume

**Table 1.** Sample information and measurement result of the furfuryl alcohol content in the authentic samples

| Sample No. | Farm | Country | Species | Variety | Processing | Furfuryl alcohol, mg/kg |
|---|---|---|---|---|---|---|
| **1198** | *Badra Estates* | India | Canephora | SLN 274 / Old Paradenia | Dry | 92 |
| **13BA001** | *Badra Estates* | India | Canephora | SLN 274 / Old Paradenia | Semi-washed | 107 |
| **13BA002** | *Badra Estates* | India | Canephora | SLN 274 / Old Paradenia | Dry | 101 |
| **13BA003** | *Badra Estates* | India | Canephora | SLN 274 / Old Paradenia | Dry | 96 |
| **13BA004** | *Badra Estates* | India | Canephora | SLN 274 / Old Paradenia | Wet | 131 |
| **13BA005** | *Badra Estates* | India | Canephora | SLN 274 / Old Paradenia | Wet | 140 |
| **13BA007** | *Badra Estates* | India | Canephora | SLN 274 / Old Paradenia | Semi-washed | 90 |
| **13BA008** | *Badra Estates* | India | Canephora | SLN 274 / Old Paradenia | Wet | 147 |
| **13BA011** | *Badra Estates* | India | Canephora | CxR | Wet | 122 |
| **13BA012** | *Badra Estates* | India | Canephora | CxR | Wet | 123 |
| **13BA014** | *Badra Estates* | India | Arabica | S 795 | Wet | 251 |
| **13BA015** | *Badra Estates* | India | Arabica | S 795 | Wet | 262 |
| **13BA016** | *Badra Estates* | India | Arabica | S 795 | Wet | 200 |
| **13BA017** | *Badra Estates* | India | Arabica | S 795 | Semi-washed | 225 |
| **13BA018** | *Badra Estates* | India | Arabica | HDTxCatuaí | Wet | 205 |
| **13BA019** | *Badra Estates* | India | Arabica | HDTxCatuaí | Wet | 270 |
| **13BA025** | *Badra Estates* | India | Arabica | Catimor (BBTC) | Wet | 224 |
| **13BA999** | *Badra Estates* | India | Arabica | mixed | Wet | 274 |
| **14BA005** | *Badra Estates* | India | Robusta | SLN 274 / Old Paradenia | Wet | 105 |
| **14BA028** | *Badra Estates* | India | Arabica | S 795 | Wet | 222 |
| **16BA011** | *Badra Estates* | India | Robusta | SLN 274 / Old Paradenia | Wet | 125 |
| **16BA021** | *Badra Estates* | India | Arabica | S 796 | Wet | 243 |
| **17BA016** | *Badra Estates* | India | Arabica | S 795 | Wet | 251 |
| **17BA037** | *Badra Estates* | India | Robusta | SLN274 / Old Paradenia | Wet | 142 |
| **13PA007** | *BB Chengappa* | India | Robusta | CxR | Wet | 134 |
| **13DU006** | *Fazendas Dutra* | Brazil | Arabica | Aramosa | Semi-washed | 177 |
| **13DU008** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 250 |
| **13DU010** | *Fazendas Dutra* | Brazil | Arabica | S 795 | Wet | 237 |
| **13DU014** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Wet | 220 |
| **13DU023** | *Fazendas Dutra* | Brazil | Arabica | Bourbon amarelo | Semi-washed | 249 |
| **13DU024** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 254 |
| **13DU025** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 220 |
| **13DU026** | *Fazendas Dutra* | Brazil | Arabica | Bourbon amarelo | Semi-washed | 250 |
| **13DU027** | *Fazendas Dutra* | Brazil | Arabica | Mundo Novo vermelho | Semi-washed | 238 |
| **13DU030** | *Fazendas Dutra* | Brazil | Arabica | Catuaí amarelo | Semi-washed | 228 |
| **13DU032** | *Fazendas Dutra* | Brazil | Robusta | Conillonvermelho | Dry | 86 |
| **13DU033** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 220 |
| **13DU040** | *Fazendas Dutra* | Brazil | Arabica | Icatu amarelo | Semi-washed | 230 |
| **13DU043** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 237 |
| **13DU044** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 240 |
| **13DU053** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 244 |
| **13DU054** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 199 |
| **13DU059** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 235 |
| **13DU102** | *Fazendas Dutra* | Brazil | Arabica | Catucaiamarelo | Semi-washed | 236 |
| **10DU100** | *Fazendas Dutra* | Brazil | Arabica | Catuaí amarelo | Semi-washed | 211 |
| **10DU101** | *Fazendas Dutra* | Brazil | Arabica | Bourbon amarelo | Semi-washed | 248 |
| **10DU102** | *Fazendas Dutra* | Brazil | Arabica | Pacamaraamarelo | Semi-washed | 220 |
| **10DU103** | *Fazendas Dutra* | Brazil | Arabica | Catuaí amarelo | Semi-washed | 271 |
| **10DU104** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 245 |
| **10DU105** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 236 |
| **10DU106** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 242 |
| **10DU107** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 231 |
| **10DU108** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 271 |
| **10DU109** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 268 |
| **10DU110** | *Fazendas Dutra* | Brazil | Arabica | Catuaí amarelo | Semi-washed | 232 |
| **10DU111** | *Fazendas Dutra* | Brazil | Arabica | Catuaí amarelo | Semi-washed | 232 |
| **1142** | *Fazendas Dutra* | Brazil | Arabica | Catuaí vermelho | Semi-washed | 216 |

capacity of 60 g and a temperature capacity of 240°C. The beans in the roasting drum were kept in motion by means of an air stream between 70 and 90% flow. Different roasting profiles were used to evaluate the influence of roasting on furfuryl alcohol content. Since the final temperature significantly influenced the formation of furfuryl alcohol, another experimental design was created to further evaluate the effect of final temperature (Tables 2-4).

**Table 2.** Experimental design for investigation influence of furfuryl alcohol formation during roasting and storage (T-Start: starting temperature, T-End: Final temperature)

| No. | Ventilation,% | T-Start, °C | T-End, °C | Duration, min | Temperature-change | Storage | Furfuryl alcohol, mg/kg |
|---|---|---|---|---|---|---|---|
| **1** | 70 | 100 | 230 | 20 | Exponentially | Beans, 2 weeks | 76.0 |
| **2** | 85 | 150 | 230 | 20 | Exponentially | 1 day | 85.1 |
| **3** | 85 | 100 | 230 | 20 | Linear | Ground, 2 weeks | 129.5 |
| **4** | 85 | 150 | 200 | 5 | Exponentially | 1 day | 75.2 |
| **5** | 70 | 100 | 230 | 5 | Linear | Beans, 2 weeks | 109.3 |
| **6** | 70 | 150 | 230 | 20 | Linear | Ground, 2 weeks | 142.4 |
| **7** | 70 | 100 | 230 | 20 | Exponentially | Ground, 2 weeks | 78.1 |
| **8** | 85 | 100 | 200 | 20 | Linear | Beans, 2 weeks | 57.1 |
| **9** | 70 | 100 | 200 | 5 | Exponentially | 1 day | 78.9 |
| **10** | 70 | 150 | 230 | 5 | Exponentially | 1 day | 200.1 |
| **11** | 70 | 150 | 200 | 20 | Exponentially | 1 day | 112.7 |
| **12** | 70 | 150 | 200 | 5 | Linear | 1 day | 56.2 |
| **13** | 70 | 150 | 200 | 5 | Exponentially | Beans, 2 weeks | 70.1 |
| **14** | 70 | 150 | 200 | 5 | Exponentially | Ground, 2 weeks | 70.2 |
| **15** | 70 | 100 | 200 | 20 | Linear | Ground, 2 weeks | 73.9 |
| **16** | 85 | 100 | 200 | 20 | Exponentially | 1 day | 98.7 |
| **17** | 85 | 100 | 200 | 5 | Linear | 1 day | 29.9 |
| **18** | 85 | 100 | 200 | 5 | Exponentially | Ground, 2 weeks | 50.8 |
| **19** | 70 | 100 | 230 | 20 | Linear | 1 day | 177.5 |
| **20** | 85 | 100 | 230 | 5 | Exponentially | 1 day | 150.7 |
| **21** | 85 | 150 | 230 | 5 | Linear | 1 day | 110.1 |
| **22** | 85 | 150 | 230 | 5 | Exponentially | Beans, 2 weeks | 147.3 |
| **23** | 85 | 150 | 230 | 5 | Exponentially | Ground, 2 weeks | 146.2 |
| **24** | 85 | 150 | 200 | 20 | Linear | 1 day | 78.3 |
| **25** | 70 | 100 | 230 | 5 | Linear | Ground, 2 weeks | 101.0 |
| **26** | 85 | 150 | 200 | 20 | Exponentially | Ground, 2 weeks | 75.0 |
| **27** | 85 | 150 | 200 | 5 | Linear | Ground, 2 weeks | 49.3 |
| **28** | 70 | 150 | 230 | 20 | Linear | Beans, 2 weeks | 119.8 |
| **29** | 70 | 100 | 200 | 5 | Linear | Beans, 2 weeks | 37.9 |
| **30** | 85 | 100 | 200 | 5 | Linear | Beans, 2 weeks | 38.5 |
| **31** | 70 | 100 | 200 | 20 | Linear | 1 day | 84.3 |
| **32** | 70 | 100 | 200 | 5 | Linear | 1 day | 47.3 |
| **33** | 70 | 100 | 200 | 5 | Exponentially | Beans, 2 weeks | 62.6 |
| **34** | 85 | 100 | 230 | 20 | Linear | Ground, 2 weeks | 139.1 |
| **35** | 85 | 150 | 230 | 5 | Exponentially | Beans, 2 weeks | 144.0 |

Since coffee beans after roasting emit $CO_2$, the roasted beans were stored at least 12 h before being ground. A Mahlkönig EK 43 machine (Mahlkönig, Hamburg, Germany) was used for grinding coffee beans. To minimize possible contamination between samples, the shaft of the grinder mill was cleaned with a brush before each grinding operation. In addition, the first 15 to 30 g of the ground samples were discarded. The grinder was set at different grinding degrees (fineness one-nine) with grind three selected for use as it gave a fineness equivalent to that of commercially-available ground coffee.

Analysis of variance (ANOVA) was conducted in order to determine if the roasting parameters had significant influence on the formation of furfuryl alcohol in coffee. For this purpose, results were obtained from roasting a sample of an Indian *C. canephora* coffee. The parameters tested included temperature at the beginning (100°C, 150°C) and end of roasting (200°C, 230°C), profile of the temperature curve (linear, exponential for 5 min, 20 min), the velocity of the air flow (70%, 85%), and the duration of storage of the coffee between the roasting and measuring the coffee samples (18 h, or 2 weeks either directly ground or stored as beans) using a D-optimal factorial design. Selected roasting profiles are shown in Supplemental Figure S1. ANOVA was conducted using Design Expert 7 (Minneapolis, MN, USA).

## Results and Discussion

### Optimization of Sample Extraction and NMR Assignment of Furfuryl Alcohol

During the development and optimization of an extraction method, it was important to select a suitable solvent offering satisfactory recoveries for all relevant analytes. Deuterated solvents such as $CDCl_3$, methanol-$d_4$, $D_2O$, acetone-$d_6$ and DMSO-$d_6$ are preferred for NMR because they do not overload the receiver with their own proton signal(s). However, the choice of solvent depends on its residual signal(s) being apart from any analyte signals. The NMR signals of $D_2O$, methanol-$d_4$ and DMSO-$d_6$ overlapped with those of furfuryl alcohol. Deuterated acetone and chloroform gave clearly isolated signals. However, deuterated acetone gave lower signal intensities for caffeine and kahweol. Based on these results, $CDCl_3$ was selected as solvent for extraction of the analytes. The use of deuterated chloroform for the extraction of furfuryl alcohol has also been reported in the literature (4, 18).

To improve the extraction process, the efficacies of mechanical shaking and ultrasonication of the coffee-solvent-slurries were compared. Ultrasonication gave 30-60% higher NMR signal intensities than mechanically shaken extracts. The completeness of the respective extractions of ground coffee was

**Table 3.** Result of the analysis of variance (ANOVA) to determine the influence of roasting parameters on the formation of furfuryl alcohol (design in Table 2)

| | Degrees of freedom | Mean square variance | *F* value | *P* value | |
|---|---|---|---|---|---|
| **Model** | 7 | 5718.65 | 7.65 | < 0.0001 | Significant |
| **Strength of the airflow** | 1 | 191.09 | 0.26 | 0.62 | |
| **Starting temperature** | 1 | 1150.66 | 1.54 | 0.23 | |
| **Final temperature** | 1 | 33 044.00 | 44.22 | <0.0001 | Significant |
| **Duration of roasting** | 1 | 323.45 | 0.43 | 0.52 | |
| **Temperature curve** | 1 | 800.96 | 1.07 | 0.31 | |
| **Duration of storage** | 2 | 1274.09 | 1.71 | 0.20 | |

**Table 4.** Experimental design to study the influence of maximum temperature on furfuryl alcohol formation. The roasting started in each case with a starting temperature of 125°C and was linearly increased to the specified temperature. The duration of the roasting was set to 10 min and the air flow was set at 70%

| No. | Final temperature, °C | Furfuryl alcohol, mg/kg |
|---|---|---|
| **1** | 180 | 32.5 |
| **2** | 180 | 45.6 |
| **3** | 210 | 96.5 |
| **4** | 210 | 81.9 |
| **5** | 240 | 170.6 |
| **6** | 210 | 93.8 |
| **7** | 180 | 42.0 |
| **8** | 240 | 167.3 |
| **9** | 240 | 170.2 |
| **10** | 240 | 171.4 |
| **11** | 225 | 147.8 |
| **12** | 195 | 64.5 |
| **13** | 218 | 112.3 |
| **14** | 188 | 47.2 |
| **15** | 233 | 177.7 |
| **16** | 240 | 182.0 |
| **17** | 180 | 49.8 |
| **18** | 240 | 162.2 |
| **19** | 180 | 39.9 |
| **20** | 210 | 78.7 |

ascertained by conducting a second extraction of the residue obtained after the first extraction.

Accurate assignment of the NMR signals of furfuryl alcohol was performed by duplicate analysis of coffee samples. In the second run, the coffee samples were spiked with a known amount of furfuryl alcohol, resulting in an increase of the signals of furfuryl alcohol. However, this method does not exclude the possibility that other substances may falsely augment the furfuryl alcohol signal. To safeguard against this, a literature search was performed for substances likely to exist in roasted coffee which have similar chemical shifts as furfuryl alcohol. The candidate structural analogues with similar resonances included furfuryl acetate, 2-furanmethanethiol, furfural and 2-methoxy-4-vinylphenol (35, 36). In order to clearly distinguish between signals, reference standards were independently measured followed by spiking the coffee with structural analogues. Figure 1 shows an NMR spectrum of the coffee samples spiked with the standards.

Furfuryl alcohol gave NMR resonances at ≈7.40 ppm while the adjacent signals ≈ 7.42 ppm and ≈ 7.36 ppm originated from furfural and furanmethanethiol, respectively. These two resonances (7.36 and 7.42 ppm) were distinctly separated from those of furfuryl alcohol. The signal from furfural overlapped with that of $CDCl_3$ (≈7.25 ppm) while the signals of 2-methoxy-4-vinylphenol do not interfere with those of furfuryl alcohol. None of the reviewed substances interfered with the quantification of furfuryl alcohol. Ethylbenzene produced two signals in the high-field and a signal in the low-field whereas TCNB gave only one signal.

The extraction and proton NMR analysis required minimal sample preparation steps. The developed extraction method utilizing $CDCl_3$ with ultrasonication was not only found to be simple but also fast.

## Influence of Roasting on Furfuryl Alcohol Formation

The formation of furfuryl alcohol in coffee may be dependent on many parameters including starting temperature, final temperature, duration of roasting, temperature profile and the duration of storage of roasted coffee. Roasting experiments identified only the maximum temperature as having a significant impact on the content of furfuryl alcohol ($P < 0.0001$) (Figure 2; Table 3). The other parameters investigated play a minor role in the formation of furfuryl alcohol. Although the influence of the other factors is minimal, a comparison of different samples with identical degree of roast when stored for different durations revealed that the content of furfuryl alcohol decreases within 2 weeks.

NMR characterization of the degradation pathway showed that furfuryl alcohol is reduced to the aldehyde, furfural, which gives a signal in the low field region. This finding on the formation of furfural from furfuryl alcohol corroborates the results of Moon and Shibamoto (22).

In another study utilizing SPME-GC-MS to monitor volatiles formed during coffee roasting, furfuryl alcohol was found to decompose and vaporize (37). Acid-catalyzed decomposition of furfuryl alcohol via polymerization to form dimers and trimers has also been reported in the literature (19, 38). The polymerization pathway is highly feasible in coffee samples since the high content of organic acids in beans creates an acidic environment. Additionally, the reaction between 2-deoxyribose and formic acid further lowers the pH favoring polymerization of furfuryl alcohol.

In general, the maximum temperature should be kept as low as possible in order to minimize the formation of furfuryl alcohol. A long, low-temperature roasting is therefore better than a short roasting at high temperatures. Nevertheless, such roasting fashion may lead to a concomitant increase of other contaminants such as acrylamide (14). There is certainly the need to confirm the results in industry-scale roasters.

## Survey of Furfuryl Alcohol in Authentic Coffee Samples

Evidently, differences in the content of furfuryl alcohol were noted in the different types and varieties of coffee. Indeed, even

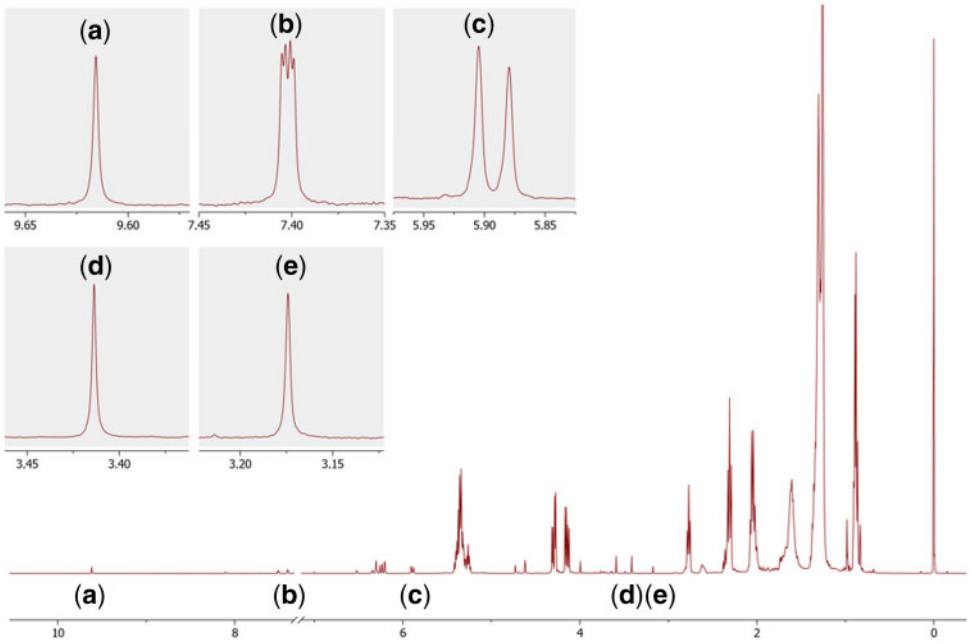


**Figure 1.** NMR spectrum of a coffee sample with assignments of analytes: a) HMF, b) furfuryl alcohol, c) kahweol, d) caffeine, e) 16-O-methylcafestol.

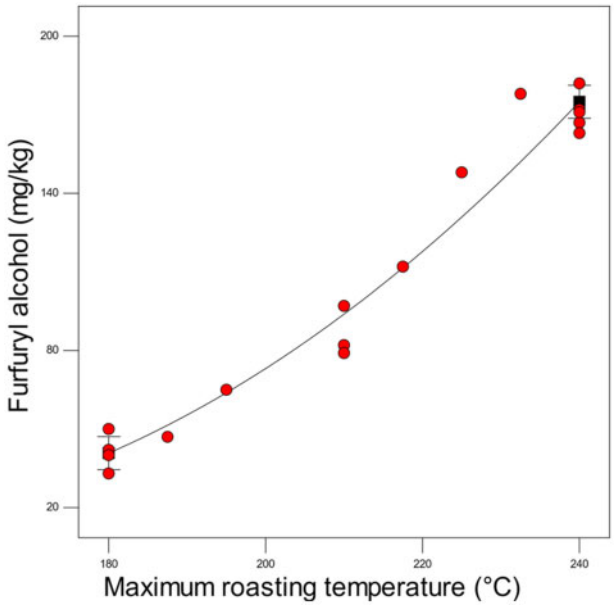


**Figure 2.** Formation of furfuryl alcohol depending on the final or maximum temperature during roasting.

the geographic origin may affect the composition of coffee since the soils the coffee grew in differ too. A comparison of coffee beans was conducted on 57 samples using the same roasting parameters.

Results show that the amounts of furfuryl alcohol in the coffee beans differ despite the same roasting conditions. The average content of furfuryl alcohol in all samples was 204 ± 28 mg/kg. The lowest content of 86 mg/kg was in a *C. canephora* sample whereas the highest content of 274 mg/kg was in a *C. arabica* sample. The sample having the highest content of furfuryl alcohol was a "monkey coffee" selection. The "monkey coffee" originates from monkeys that pick and eat ripe coffee cherries. The monkeys spit out the hard coffee beans, which are then collected by farm workers. It is presumed that monkey coffee's particular flavor is due to enzymes in the saliva of the apes, quite possibly these enzymes also promote the formation of furfuryl alcohol.

Overall, *C. canephora* coffee formed significantly less furfuryl alcohol compared to *C. arabica* coffee (Arabica mean 236 mg/kg, Canephora mean 116 mg/kg). The total content of furfuryl alcohol in the roasted coffee arises from several pathways. On the one hand, furfuryl alcohol is formed during several heat-induced reactions of sugars such as sucrose, fructose and glucose, and on the other hand also from chlorogenic acid (12, 22, 23, 39). *C. arabica* coffee contains more sugar than *C. canephora* coffee whereas *C. canephora* coffee contains more chlorogenic acid than *C. arabica* coffee. In green coffee, the content of chlorogenic acid is greater than that of sucrose (2). The significantly higher level of furfuryl alcohol in *C. arabica* coffees shows that sucrose or monosaccharides already contained in coffee may have a greater impact on the formation of furfuryl alcohol than the existing organic acids.

## Risk Assessment of Furfuryl Alcohol in Coffee

Evaluation of spectra of coffee samples analyzed at CVUA-Karlsruhe in the years 2015-2017 was carried out to obtain comprehensive data on the occurrence of furfuryl alcohol on the German market. The coffee samples whose NMR spectra were evaluated were either ground coffee or coffee beans but also included coffee capsules, pads or soluble coffee samples. The mean content of furfuryl alcohol for the 582 samples of 55.6 mg/kg was significantly less than the previously measured value of 251 mg/kg (18). One reason, among others, may be the inclusion of pre-ground samples, whose furfuryl alcohol content might be lowered by evaporation during storage. Longer storage-time leading to a decrease is also known for other coffee contaminants such as acrylamide (14).

In animal studies, tumors of the renal tubular epithelium were found in mice fed orally with furfuryl alcohol (40). Assuming a daily consumption of one cup of coffee, an exposure of 0.01 mg/kg bw of furfuryl alcohol should be expected. The resulting margin of exposure (MOE) will be approximately 5000 (for details on calculation, see (14)). This worst-case scenario estimation of exposure assumes a 100% extraction of furfuryl alcohol from the beans to the coffee drink. The MOE estimates the concentration of a substance in animals that would lead to negative consequences as a result of exposure to the agent. Only a MOE of over 10 000 is regarded as very low risk for humans (41). However, the MOE does not take into account the mechanism of action and bioavailability for humans. Despite this uncertainty, tumorigenic effects of ethanol cannot be ignored in persons consuming both coffee and alcoholic beverages (42). Ethanol may potentiate the tumorigenic effects of furfuryl alcohol by delaying the excretion via saturation of the metabolic enzymes, alcohol and aldehyde dehydrogenases (42). The exposure of furfuryl alcohol is not only limited to coffee, but also many other heat-treated foods (18, 21) contain the carcinogen. However, the content of furfuryl alcohol in coffee is still judged too small to pose a risk for cancer, and coffee itself has been typically not associated with increasing the cancer risk in epidemiological studies (14).

## Conclusions

The investigations of the roasting process showed that only the maximum temperature has a significant influence on the formation of furfuryl alcohol. The content of furfuryl alcohol increases with temperature. Among the coffee species, *C. arabica* contains more furfuryl alcohol than *C. canephora*. This is attributable to the higher sucrose content in *C. arabica*. In addition, dry processing showed a lower production of furfuryl alcohol as opposed to the wet processing.

The PULCON NMR methodology was found to be fit for purpose as it enabled a simple, rapid and accurate determination of constituents of coffee. The PULCON quantification gives the high analytical throughput required for routine screening of coffee not only for food fraud detection purposes but also for detection of several important health-relevant contaminants.

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## Supplemental Information

Supplemental information is available on the *J. AOAC Int.* website.

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