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Fully automatic quantitation of eight different metabolites in coffee using ^{1}H-NMR spectroscopy and the PULCON methodology

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Abstract

Background: Coffee contains a plethora of constituents with some of them being especially important either due to their physiological effects or as quality markers. As quantitative proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) has been established as a fast and reliable analytical tool its application was evaluated for the simultaneous quantitation of lactic acid, acetic acid, formic acid, caffeine, caffeoylquinic acid (CQA) isomers, *N*-methylpyridinium, trigonelline, and 5-hydroxymethylfurfural (HMF) in aqueous extracts of roasted *Coffea arabica* samples.

Results: Simultaneous quantitative determination was achieved by an automated analysis based on the PULCON methodology (pulse length-based concentration determination). The method was validated regarding linearity, accuracy, precision, limit of detection (LOD), and limit of quantitation (LOQ). Recovery rates were between 76% (CQA) and 116% (HMF), and precision was between 1.7% (caffeine) and 10.3% (HMF). The LOD varied between 0.06 g/kg (HMF) and 1.35 g/kg (caffeine and CQA), with the LOQ being between 0.22 g/kg (HMF) and 4.87 g/kg (CQA). To verify the results of the ^{1}H-NMR method, caffeine, trigonelline, HMF, 3-CQA, 4-CQA, and 5-CQA were additionally quantitated by HPLC-DAD and the results were compared. The described ^{1}H-NMR method was additionally applied to coffee samples that contained different coffee defects. Results showed only slight changes in the concentrations of the analytes by adding defective beans to defect-free coffee.

Discussion: The developed 1H-NMR approach was proven to be fast (30 min), reliable, and precise. Thus, it is well suited to analyze several coffee constituents of interest in a large number of samples in, for example, quality control.

KEYWORDS
^{1}H-NMR spectroscopy, coffee, metabolites, PULCON, qNMR, quantitation

INTRODUCTION

Coffee is a worldwide consumed beverage usually prepared from roasted coffee seeds from the *Coffea* plant. According to the International Coffee Organization (ICO), 1.7 million 60 kg bags of coffee were consumed worldwide from 2020 to 2021, with coffee consumption being highest in Europe, followed by Asia and North America.[1] This demonstrates that coffee has a high economic status and is one of the most legally traded products worldwide.[2,3] The coffee production is dominated by the coffee species *Coffea arabica* L. (arabica), which

occupies a percentage of 57%, followed by a production of 43% *Coffea canephora* Pierre ex A. Froehner (canephora).[4] Green coffee beans are produced in more than 70 countries, including cultivation, harvesting, and post-harvest treatment.[5] The green coffee beans are then exported to the consuming country, where the roasting process usually takes place. During the roasting process, the coffee beans undergo several physical and chemical changes resulting in flavor rich, roasted coffee. The quality of a cup of coffee is influenced by many factors, including the physical and chemical characteristics of the green coffee beans and the conditions of the roasting process.[6] Due to a lack of minor elements, viral or bacterial infections of roots and fruits, attacks by fungi, insects, or parasites and environmental stress, the coffee plant is subject to a wide range of diseases, which may have a negative effect on the quality of coffee.[3]

Roasted coffee is a complex mixture of flavor (aroma and taste) compounds, lipids, carbohydrates, nitrogenous compounds, vitamins, minerals, alkaloids, and phenolic compounds.[7,8] Caffeine, which is formed as part of the purine metabolism, has a stimulating effect on the human nervous system.[7,9,10] Other nitrogenous compounds such as trigonelline and its thermal degradation product *N*-methylpyridinium (NMP) may have effects on the cellular energy metabolism, chemopreventive activity, and antioxidant capacity.[10,11] Chlorogenic acids (CGA), which are the main phenolic components in coffee, are antioxidants, too, and show anti-inflammatory and neuroprotective activities.[12–14] In roasted coffee, caffeoylquinic acids (CQA), consisting of the structural isomers 5-CQA, 4-CQA, and 3-CQA, are the dominant CGA.[8] Organic acids contribute to the sour taste of coffee. The main organic acids in coffee are citric acid, acetic acid, formic acid, malic acid, lactic acid, and quinic acid. Quinic acid is mainly bound to hydroxycinnamic acid isomers to form CGA.[7,15] In addition to constituents with potential health benefits, there may be compounds of concern in roasted coffee depending, among others, on the roasting conditions. These compounds include acrylamide, furfuryl alcohol, and 5-hydroxymethylfurfural (HMF), some of which have been classified as possible or probable human carcinogens by the International Agency for Research and Cancer (IARC).[16,17] Also, excessive caffeine consumption may result in increased blood pressure and insomnia.[18] Thus, in order to check the quality of the roasted coffee and monitor the components of concern, a quantitative determination of the aforementioned constituents is essential.

There are many studies regarding the quantitative determination of several coffee constituents using chromatographic, spectroscopic, and spectrometric methods, which have been published over the last decades.[19–28] Several of these methods are based on high performance liquid chromatography (HPLC) using different detectors for the quantitation of quinic acid, nicotinic acid, trigonelline, NMP, caffeine, 5-CQA, HMF, furfural, and acrylamide.[22–28] Organic acids are often quantitated using ion chromatography.[19] Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) was applied to quantitate CQA isomers, formic acid, acetic acid, caffeine, trigonelline, NMP, and HMF in lipophilic roasted coffee extracts.[20,25] In addition, caffeine, formic acid, trigonelline, and HMF were quantitated in instant coffees using ^{1}H-NMR.[21] As ^{1}H-NMR is a primary analytical technique, absolute and relative simultaneous quantitation of several ingredients is possible using either an internal or external standard.[29,30] By using the method of pulse length based concentration determination (PULCON), concentrations are calculated by correlation of the absolute integral of the analyte signal and an external standard.[31] By measuring the standard separately (so called reference solution), a possible overlap of the signals or interactions between the standard substance and analytes are avoided. Signal influences, which may occur due to different physical properties of the solutions, are solved by the principle of reciprocity.[31] To the best of our knowledge, there is no comprehensive study to quantitate different ingredients in an aqueous extract of roasted coffee using ^{1}H-NMR.

Thus, we present a new method for the simultaneous quantitation of lactic acid, acetic acid, formic acid, caffeine, trigonelline, NMP, CQA, and HMF in aqueous coffee extracts using ^{1}H-NMR following a simple and quick sample preparation.

MATERIALS AND METHODS

Samples

A total of 603 arabica samples were investigated. In addition, coffee samples containing 17 different green coffee defects were analyzed to study whether these defects affect concentrations of the considered metabolites. Defects were classified according to the Green Coffee Association of New York.[32] Here, the number of defects is evaluated in 300 g of coffee. Depending on the type and amount of defective beans in defect-free coffee, a distinction is made between the classification levels NY0 – NY8 with NY8 representing the lowest quality level. The following defects at the quality levels NY2, NY4, and NY6 were investigated: broken beans, dark green beans, eaten beans, well eaten beans, black beans, faded beans, bleached beans, sour beans, pulper cuts, unripe beans, malformed, quaker, pods, parchment, husks, shells, and twigs. Coffee samples were either taken by official food inspectors of the German Federal State of Baden Württemberg (Karlsruhe, Germany) or provided by Coffee Consulate (Mannheim, Germany) from 2019 to 2022. Roasted coffee beans were ground to a grain size of 0.3 mm with a coffee mill (EK43, Mahlkönig, Bachenbülach, Switzerland).

Chemicals and reagents

Reagents and chemicals were of analytical or HPLC grade. Deuterium oxide (99.9 atom% D) was from Deutero (Kastellaun, Germany). HMF (≥ 97%), 5-CQA (≥ 97%), sodium acetate (≥98.5%), and trigonelline hydrochloride (≥ 97.5%) were purchased from Carl Roth (Karlsruhe, Germany). Citric acid monohydrate (≥ 99.5%), orthophosphoric acid (85%), 3-CQA (≥ 95%), 4-CQA (≥ 98%), and sodium dihydrogen phosphate monohydrate (≥ 99%) were from Merck (Darmstadt, Germany). Caffeine (99%), sodium formate (99%), sodium lactate (98%), NMP iodide (≥ 97%), and 3-(trimethylsilyl)-propionic acid-*d4* sodium salt (TSP) (98 atom% D) were purchased from Sigma-Aldrich (Steinheim, Germany).

Buffer was prepared by dissolving 138 g sodium dihydrogen phosphate monohydrate in 1000 mL of H_2O and adjusting the pH value to 6.0 with orthophosphoric acid.

Sample preparation, optimization of extraction conditions

To obtain optimal extraction conditions for quantitation of the eight coffee metabolites, different proportions of sample to extraction solvent were tested first. For this purpose, different amounts (0.1, 0.2, 0.6, and 1.2 g) of ground coffee samples of different roasting degree (light and dark) were extracted using 8 mL of extraction solvent (H_2O). After shaking the samples on a test tube shaker (1600 U/min, Multi Reax, Heidolph, Schwabach, Germany) for 20 min, the suspension was passed through a membrane filter (GF/PET, 0.25 µm). The filtered coffee extracts (600 µL) were mixed with 100 µL of buffer solution and 70 µL of internal standard solution (deuterium oxide containing 1 g/L TSP). For ^{1}H-NMR measurement (see below), an aliquot of 600 µL was transferred into an NMR tube (Deuquant, glass, o.d. 4.966 ± 0.004 mm, i.d. 4.116 ± 0.004 mm, length 17.78 cm, Deutero, Kastellaun, Germany). Also, different extraction temperatures (room temperature (RT), 30, 40, 50, 60, and 70°C were tested, using, 0.2 g of the ground coffee sample, 8 mL of H_2O, and an extraction time of 20 min. In addition, different extraction times (20, 30, 60, 90, 120, 150, and 180 min) were tested. For this purpose, 0.2 g of the ground coffee sample was extracted with 8 mL of H_2O at RT. As a result of this optimization process, ground coffee samples (0.2 g) were extracted at RT for 20 min using 8 mL of H_2O.

^{1}H-NMR spectroscopy

All ^{1}H-NMR spectra were acquired at 300 K on a Bruker 400 MHz AVANCE III HD NanoBay spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) equipped with a 5-mm BBI (broadband inverse) probe and a Bruker automatic sample changer Sample Xpress (Bruker BioSpin MRI GmbH, Ettlingen, Germany). All samples were temperature equilibrated for 5 min. ^{1}H-NMR spectra were acquired using an optimized water suppression 1D noesygppr1d pulse sequence with a D7 delay. Here, D7 is an additional relaxation delay in the Bruker noesy1d pulse sequence, which is applied without radiation of the presaturation pulse to avoid temperature increase of the sample. Mixing time was 0.01 s. The NMR spectra were acquired with 98 k time domain data points, 128 scans, four dummy scans, spectral width of 20.56 ppm, acquisition time of 5.98 s, and a receiver gain of 79. The relaxation delays D1 and D7 were each 4 s. Spectra were recorded in the baseopt mode and for each sample, the 90° pulse width was automatically estimated. Acquired data were processed with Bruker BioSpin Topspin software (version 3.7, Bruker BioSpin MRI GmbH) using zero filling to 256 k data points. An exponential window function (line broadening factor of 0.3 Hz) was applied, followed by Fourier transformation. Spectral phasing and baseline correction were carried out automatically. The spectra were aligned against the TSP signal at 0.00 ppm. All spectra were recorded with the same parameters and under the same conditions. To ensure the quality of the spectra, the full width at half maximum of the TSP signal was determined. A limit of 1.2 Hz was set; if this was exceeded, the measurement or sample preparation was repeated.

Quantitation

The concentrations of lactic acid, acetic acid, caffeine, CQA, formic acid, NMP, trigonelline, and HMF were determined based on the PULCON principal.[31,33,34]

To apply the PULCON principle, a so called ERETIC factor is needed, which correlates the intensities of the signals in two separately measured solutions (the reference solution and the sample solution). To calculate the ERETIC factor,[33,34] an aqueous (aq.) solution containing citric acid (4.2 g/L) and lactic acid (0.92 g/L) was prepared. A 600 µL-aliquot of this solution was mixed with 100 µL of buffer solution and 70 µL of the internal standard solution (deuterium oxide containing 1 g/L TSP), generating a dilution factor of 0.78. This reference solution was used comparable with an external standard to calculate the ERETC factor and was measured in each sample series. The doublet at $\delta = 1.32–1.49$ ppm for lactic acid and the doublet at $\delta = 2.90–3.03$ ppm for citric acid were used to calculate the ERETIC factor:

$$\mathrm{ERETIC} = \frac{I_{Ref} \bullet SW_{Ref} \bullet MW_{Ref}}{SI_{Ref} \bullet c_{Ref} \bullet N_{Ref} \bullet DF} \quad (1)$$

I_{Ref} is the absolute integral (Ref = reference substance), SW_{Ref} is the spectral width (20.56 ppm), MW_{Ref} is the molecular weight (90.08 g/mol for lactic acid and 192.12 g/mol for citric acid), c_{Ref} is the concentration (0.92 g/L for lactic acid and 4.2 g/L for citric acid), N_{Ref} is the number of protons generating the selected signal (three for lactic acid and two for citric acid), DF is the dilution factor (0.78). SI_{Ref} is the size of the real spectrum, which shows the number of data points after the Fourier transformation (256 k). The average ERETIC factor was used for the quantitation of the analyte concentrations using the following equation:

$$c_X = \frac{I_X \bullet SW_X \bullet MW_X \bullet P1_X \bullet V}{SI_X \bullet ERETIC \bullet N_X \bullet P1_{Ref} \bullet DF \bullet GW} \quad (2)$$

where Ix is the absolute integral (x = analyte), SW_x is the spectral width (20.5617 ppm), MW_X is the molecular weight, SI_X is the size of the real spectrum (256 k), ERETIC is the average ERETIC factor (see Equation 1), N_X is the number of protons generating the selected signal, DF is the dilution factor (0.78). V and GW are the volume of the extraction solution (0.008 L) and the weighed portion of the coffee sample (0.2 g). $P1_X$ and $P1_{Ref}$ are the 90° pulse width for the reference solution and the sample.

The calculation of the concentration was performed automatically using the software MATLAB 2019b (The Math Works, Natick, MA, USA). The routine includes the import of the ^{1}H-NMR spectra, the extraction of data points, baseline correction, integration, quantitation according to the PULCON principle, and reporting of results as excel

files. For overlapping signals of lactic acid, acetic acid, caffeine, and CQA, a line-shape fitting algorithm was used for integration as described by Soininen et al. and Teipel et al.[35,36] The integral was calculated by fitting a Voigt function to the signal. By varying the characteristics for a signal, such as chemical shift, width, multiplet structure, or coupling constants, the Voigt function can be optimally adapted to the respective signal.[36]

Because it was not possible to quantitate the chlorogenic acids 3-CQA, 4-CQA, and 5-CQA separately, the CQA sum concentration was calculated based on the parameters for 5-CQA.

Validation

The validation process contained testing of linearity, accuracy, precision, and determination of the limit of detection (LOD) and the limit of quantitation (LOQ). In order to check linearity and accuracy and to determine LOD and LOQ, aqueous stock solutions for each analyte were prepared, and weighed coffee sample were spiked using these stock solutions. Sample preparation was carried out as described above. The concentrations of the stock solutions were 0.9 g/L for lactic acid, 1.0 g/L for acetic acid, 1.1 g/L for formic acid, 3.8 g/L for caffeine, 1.6 g/L for 5-CQA, 1.4 g/L for NMP, 2.1 g/L for trigonelline, and 0.2 g/L for HMF. Spiking experiments were performed at eight different concentration levels for each analyte. Concentrations of the analytes that were added to the coffee samples were 0.23–9.12 g/kg for lactic acid, 0.91–12.8 g/kg for acetic acid, 2.35–32.9 g/kg for caffeine, 4.04–48.5 g/kg for 5-CQA, 0.87–6.99 g/kg for formic acid, 0.21–26.2 g/kg for NMP, 1.87–26.2 g/kg for trigonelline, and 0.20–1.63 g/kg for HMF. Linearity was checked by regression analysis of the eight different concentrations. Accuracy was evaluated by calculation of the recovery range. LOD and LOQ were determined according to DIN 32645 calibration graph method.[37]

As a measure for precision, sample preparation and measurement of one coffee sample was carried out five times on two consecutive days. In addition, sample preparation and measurement of the same coffee sample was performed five times by different operators. Concentrations of the analytes were determined as described above and standard deviations were calculated.

Reference HPLC method

Sample preparation

Roasted, ground coffee samples (0.05 g) were extracted at room temperature with 8 mL of water. After shaking the samples on a test tube shaker (1600 U/min, Multi Reax, Heidolph, Schwabach, Germany) for 60 min, the suspension was passed through a membrane filter (GF/PET, 0.25 µm) and analyzed by HPLC coupled with a diode array detector (HPLC-DAD, Agilent Technologies Inc., Waldbronn, Germany).

HPLC-DAD conditions

HPLC analysis was carried out using a binary pump unit, degasser, column oven, auto sampler, and a DAD detector (Model 1200 series, Agilent Technologies Inc.). Chromatographic separation was achieved on a Synergi™ polar-RP 80 Å column (250 × 2 mm, 4 µm; Phenomenex, Aschaffenburg, Germany). The mobile phase was prepared from 0.3% aqueous formic buffer, pH = 2.4 (A) and methanol (B). The elution gradient was 100% A for 0–5 min, linearly decreased to 1% A within 15 min (5–20 min), kept at 1% A between 20 and 25 min, linearly increased to 100% A within 30 sec (25–25.5 min) and finally kept at 100% A between 25.5 and 35 min. The injection volume was 5 µL, the flow rate was 0.3 mL/min, and the temperature of the column was set at 35°C. Trigonelline, HMF, and caffeine were monitored at 270 nm, 3-CQA, 4-CQA, and 5-CQA at 330 nm.

Quantitation

Quantitation was performed using external calibration curves. Solutions of standard compounds within the following concentration ranges were prepared: 0.09–0.19 mg/mL for caffeine, 0.04–0.18 mg/mL for trigonelline, 0.03–0.17 mg/mL for 5-CQA, 0.01–0.14 mg/mL for 4-CQA, 0.01–0.16 mg/mL for 3-CQA, and 0.02–0.17 mg/mL for HMF. Concentrations of 3-CQA, 4-CQA, and 5-CQA were reported as sum of CQA.

Validation

Validation was carried out for the analytes caffeine, trigonelline, 3-CQA, 4-CQA, 5-CQA, and HMF. To check linearity, accuracy, precision, LOD, and LOQ, aqueous stock solutions of each analyte were prepared. Weighed coffee samples were spiked with the stock solutions, and sample preparation was carried out as described above. Spiking experiments were performed at eight different concentration levels for each analyte within the concentration ranges of 16.7–33.0 g/kg for caffeine, 6.76–30.0 g/kg for trigonelline, 7.19–22.3 g/kg for 3-CQA, 6.19–19.7 g/kg for 4-CQA, 9.66–27.2 g/kg for 5-CQA, and 3.42–25.8 g/kg for HMF. Linearity, accuracy, precision, LOD, and LOQ were evaluated as described above.

Determination of caffeine by HPLC

Sample preparation

Caffeine was additionally analyzed according to the German reference methodology for the determination of caffeine in coffee and coffee

products.[38] This an official, standardized, validated analytical method in Germany to be used by food surveillance authorities. In brief, roasted, ground coffee (1 g) was mixed with 5 g of magnesium oxide and 100 mL of water in a 250-mL volumetric flask. The mixture was heated at 90° for 20 min with constant and moderate shaking. After tempering, the solution in the volumetric flask was made up with water to the mark, passed through a membrane filter (0.45 µm, PTFE), and analyzed by HPLC-DAD.

HPLC-DAD conditions

Determination of caffeine was carried out using the same HPLC system as described above. Also, a Synergi™ polar-RP 80 Å column (250 × 2 mm, 4 µm; Phenomenex) was used. The mobile phase was an aqueous solution containing 24% methanol, the flow rate was 1 mL/min. The injection volume was 10 µL, and caffeine was monitored at 272 nm.

Quantitation

Determination of the caffeine concentration was done using an external calibration curve within the concentration range of 25–200 mg/L.

RESULTS AND DISCUSSION

Optimization of extraction conditions

In order to optimize the extraction conditions for lactic acid, acetic acid, formic acid, caffeine, CQA, NMP, trigonelline, and HMF to be measured by NMR, the ratio of coffee sample amount to extraction solvent was tested first. Amounts of 0.1, 0.2, 0.6, and 1.2 g of ground coffee were each extracted using 8 mL of extraction solvent. Comparison of the resulting ^{1}H-NMR spectra showed that extraction of larger sample amounts led to shifts of the caffeine signals (Figure S1). These shifts are possibly attributed to the shelf-association of caffeine and the formation of a concentration dependent π complex with CQA.[39,40] Since shifting signals confound the automatic quantitation process, the ratio of sample amount to extraction solvent was chosen as small as possible.

Then, analytes were analyzed by NMR following different extraction times and extraction temperatures. Because the amount of HMF was below the LOQ, this analyte was no longer considered in the optimization process. By using an extraction time of 20 min, the analytes were already extracted reproducibly from the coffee matrix. A longer extraction time did not result in higher yields of the analytes (Figure S2A). Also, extraction at RT was demonstrated to be reproducible (Figure S2B). An elevated extraction temperature of 70 °C resulted in slightly higher concentrations (about 5%) only for the analytes caffeine, CQA, and NMP compared with the extraction at RT (Figure S2B). Using higher temperatures such as 90°C (as used in the extraction protocol of the German reference methodology for the determination of caffeine in coffee and coffee products) resulted in considerably lower concentrations of CQA if compared with the extraction at RT (data not shown). Chemical instability of 5-CQA in the presence of water and high temperatures has previously been described by Dawidowicz and Typek.[41]

^{1}H-NMR analysis

The chemical structures of the compounds quantitated by ^{1}H-NMR are shown in Figure 1. The integration parameters that were used to

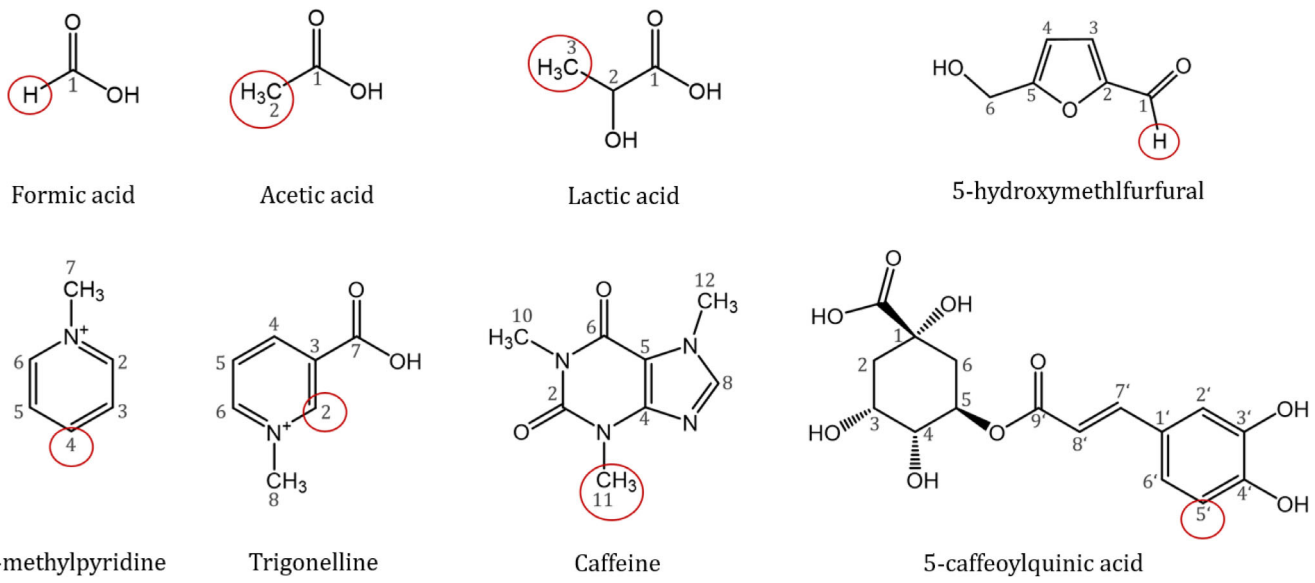

FIGURE 1 Chemical structures of formic acid, acetic acid, lactic acid, 5-hydroxymthylfurfural (HMF), N-methylpyridinium (NMP), trigonelline, caffeine, and 5-caffeoylquinic acid (5-CQA). The protons that were used for ^{1}H-NMR-based quantitation are circled.

quantitate the compounds are listed in Table S1. Figure 2 presents a ^{1}H-NMR spectrum of an aqueous coffee extract, in which the signals used for quantitation are marked. Signal assignment was performed by chemical interpretation and spiking experiments with reference substances.

The protons of CQA generate several signals in the range of 1.19–7.64 ppm. Signals that occur as doublets between 6.40 and 7.64 ppm represent the aromatic protons of caffeic acid. Protons of quinic acid produce a doublet of doublet at 3.89 ppm and multiplets in the range of 1.96–2.24 ppm. The multiplet at 5.34 ppm is shifted low-field due to the ester bond to caffeic acid. The stereoisomers 3-CQA and 4-CQA generate signals very similar to those produced by 5-CQA. Although almost all signals have slightly different chemical shifts relative to the signals of 5-CQA, they largely overlap. Differently, there is no chemical shift difference for the signals representing the H5' protons of 5-CQA, 4-CQA, and 3-CQA; all produce the doublet at 6.94 ppm, respectively. Since there are no isolated signals for the individual CQA regioisomers, CQA isomers were determined as sum parameter by integration of the doublet at 6.94 ppm.

For quantitation of the other compounds, nonoverlapping signals with the highest intensity were selected. These were the doublet at 1.33 ppm for lactic acid, the singlets at 1.93 and 8.45 ppm for acetic acid and formic acid, respectively, the singlet at 3.34 ppm for caffeine, the singlet at 9.12 ppm for trigonelline, the triplet at 8.54 ppm for NMP and the singlet at 9.46 ppm for HMF. However, the signals of lactic acid, acetic acid, caffeine, and CQA partially overlapped with matrix signals, impeding trapezoidal integration via MATLAB. Thus, a line-shape fitting algorithm was adjusted to the signals and applied to determine their integrals. The resulting curves, fitted to the signals by a Voigt function, are shown in Figure S3.

The relaxation process of the proton spins was studied by monitoring the signal areas depending on the relaxation delay (Table S2). With a relaxation delay of 8 s used here, protons of lactic acid, caffeine, CQA, NMP were completely relaxed (Figure S4). Due to the extended relaxation time, protons of acetic acid, formic acid, and trigonelline were not completely relaxed at the repetition time used (Figure S4). Therefore, correction factors were calculated for the ^{1}H nuclei of these compounds by dividing the integral of completely relaxed protons at a relaxation delay of 40 s by the integral at the relaxation delay of 8 s. This resulted in correction factors of 1.1 for acetic acid and trigonelline, and 1.2 for formic acid, which were used for quantitation. For HMF, calculation of the quotient of these signal integrals would result in a correction factor of 1.8. However, the integral of the signal of HMF did not increase steadily with the relaxation delay. These irregular fluctuations of the signal areas were attributed to a low signal to noise ratio (S/N) of <18, and the correction factor for HMF was discarded.

Validation was performed by analyzing the parameters linearity, LOD, LOQ, accuracy, and precision. Regression of the determined

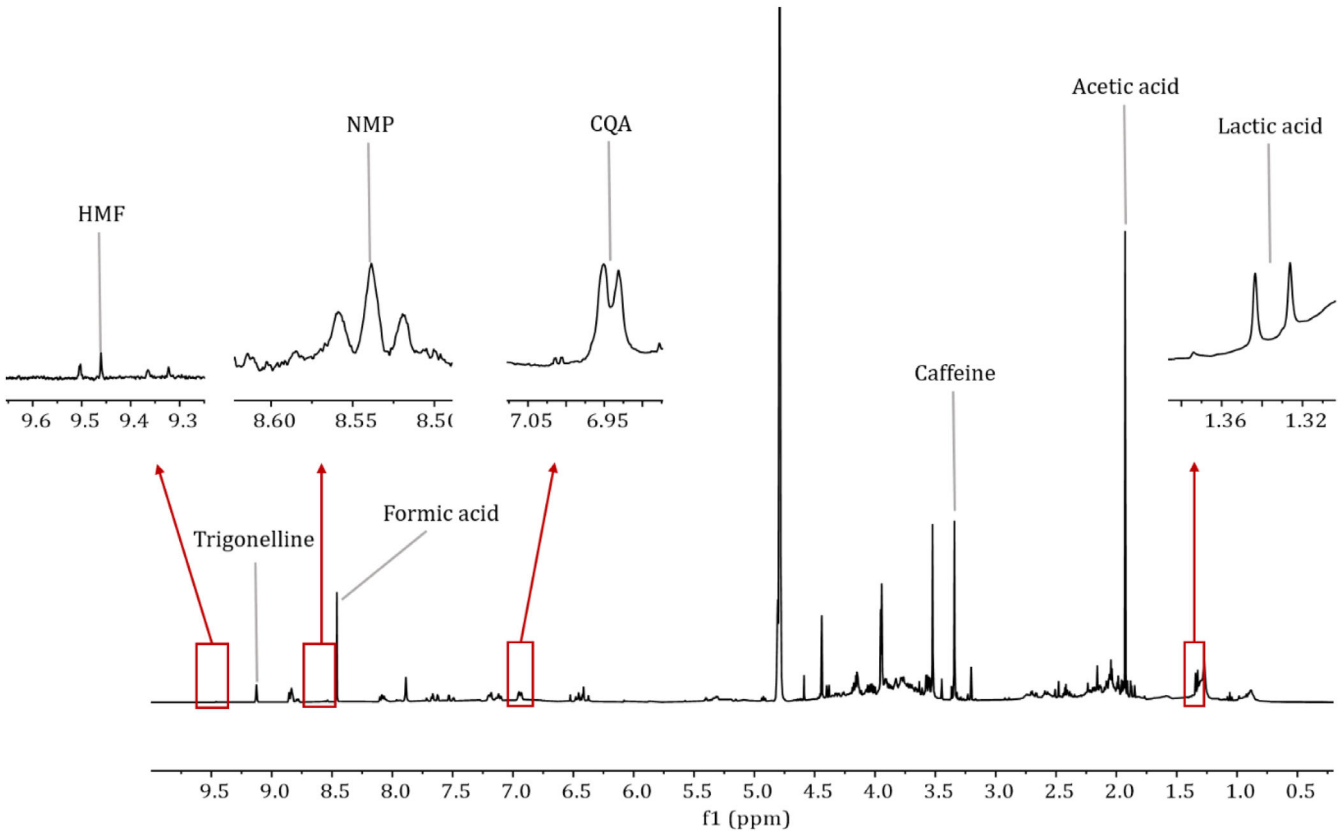

FIGURE 2 ^{1}H-NMR spectrum of an aqueous coffee extract recorded in H_2O/D_2O (9/1, *v*/*v*) using a 400 MHz NMR spectrometer. Chemical shifts were calibrated using the TSP signal at $\delta_H = 0$ ppm. The signals used to quantitate the eight compounds are indicated. Signals of 5-hydroxymthylfurfural (HMF), N-methylpyridinium (NMP), caffeoylquinic acid isomers (CQA), and lactic acid are zoomed in.

concentration against the theoretical concentration indicated a linear correlation in the concentration range tested (Table 1, Figure S5). Linear correlation for all analytes was confirmed by a residual analysis, where all residuals were scattered around the value zero. LOD and LOQ are also listed in Table 1. Del Campo et al. determined slightly lower LOQ values for the analytes formic acid, caffeine, and trigonelline and a comparable LOQ for HMF.[21] However, they investigated soluble coffee and applied a different procedure for calculating the LOQ.

Accuracy was proven based on the recovery of the analytes at different concentrations resulting from spiking weighed coffee samples with an analyte stock solution. The recovery values for the analytes lactic acid (96.3%–102.7%), acetic acid (96.2%–108.8%), formic acid (97.4%–103.9%), and trigonelline (101.9%–105.1%) were in a good range and proved the accuracy of the method for these analytes. For CQA and caffeine, lower recovery values of 76.2%–80.6% and 84.0%–86.1% were determined. This might be due to nonquantitative extraction of these compounds during sample preparation or an insufficient description of the signal structure by the Voigt function. In contrast, the recoveries of HMF (105.6%–116.2%) and NMP (103.0%–110.3%) were slightly higher, which could be due to a relatively low S/N of the signals.

Precision of the method was verified by performing sample preparation and measurement of a coffee sample multiple times on different days by two people. For multiple sample preparations and measurements performed, the coefficients of variation obtained were 2.3% for lactic acid, 5.4% for acetic acid, 2.5% for formic acid, 1.7% for caffeine, 2.9% for CQA, 1.6% for trigonelline, 2.5% for NMP, and 10.3% for HMF.

Reference HPLC method

An HPLC chromatogram of an aqueous coffee extract containing the quantified analytes is shown in Figure 3. Peak assignments were performed by spiking experiments. Recovery values for trigonelline (100.0%–101.4%), HMF (82.5%–98.0%), 3-CQA (95.2%–99.2%), 4-CQA (90.5%–108.5%), 5-CQA (94.0%–100.9%), and caffeine (90.8%–93.8%) were in an acceptable range, demonstrating adequate accuracy. For both testing linearity and determination of LOD and LOQ, regression of the determined concentration against the theoretical concentration was performed. Coefficients of determination (Table 2, Figure S6) and residual analysis indicated a linear correlation in the concentration range for all analytes tested. Determination of LOD and LOQ (Table 2) resulted in values similar to those of the ^{1}H-NMR method. This result was surprising as HPLC-DAD is generally considered more sensitive than ^{1}H-NMR. However, this result may not be due to the final analytical method but due to different sample preparation procedures. For HMF, in particular, the LOQ was very high with a value of 1.41 g/kg, which might be due to tailing of the HMF peak. Gant et al. also quantitated trigonelline, HMF, 5-CQA, and caffeine in coffee samples by HPLC-DAD and determined considerably lower values for the LOQ.[22] However, Gant et al. applied a different sample preparation and a different procedure for calculating the LOQ.

Determination of precision data resulted in coefficients of variation of 5.1% for trigonelline, 4.8% for HMF, 2.8% for 3-CQA, 1.5% for 4-CQA, 3.4% for 5-CQA, and 2.1% for caffeine (multiple sample preparations and measurements of one coffee sample). For comparability, determined concentrations of 3-CQA, 4-CQA, and 5-CQA were summed up and reported as sum parameter.

TABLE 1 Validation results of the ^{1}H-NMR method considering linearity, limit of detection (LOD), and limit of quantitation (LOQ) for the eight analytes by regression of the determined concentration against the concentration actually present.

Analyte	R^2	LOD [g/kg]	LOQ [g/kg]
Lactic acid	0.9997	0.12	0.43
Acetic acid	0.9933	0.97	3.33
Formic acid	0.9984	0.36	1.27
Caffeine	0.9997	1.35	4.80
CQA	0.9990	1.35	4.87
NMP	0.9991	0.21	0.77
Trigonelline	0.9996	0.45	1.64
HMF	0.9984	0.06	0.22

Note: R^2: coefficient of determination, N = 8 in all equations.

Analysis of caffeine by the German reference method

In 39 coffee samples, caffeine was determined using both the NMR method and the HPLC-based German reference method for the determination of caffeine in coffee and coffee products. This was deemed necessary as the NMR method resulted in a comparably low recovery for caffeine. Comparison of caffeine concentrations as determined by both methods showed lower contents when determined by ^{1}H-NMR. The differences can be explained due to different sample preparation procedures and indicate a non-quantitative extraction of caffeine using the NMR sample preparation procedure. The concentrations of caffeine determined using NMR were lower by a factor of 1.2 ± 0.03 for all samples tested. Because the factor was sufficiently stable among 39 samples, this factor was used for further calculations to adjust the caffeine contents that were measured by NMR.

Comparison of NMR and HPLC data

Seventy-eight coffee samples were analyzed using both the ^{1}H-NMR method and the HPLC-DAD method, and the determined concentrations were compared. Figure 4 shows the concentrations of trigonelline, CQA, and caffeine of all samples determined by both methods. The concentrations of HMF determined by HPLC-DAD were below the LOQ. In the same samples determined by ^{1}H-NMR, concentrations of HMF were in the range of 0.22–0.44 g/kg. For the analytes

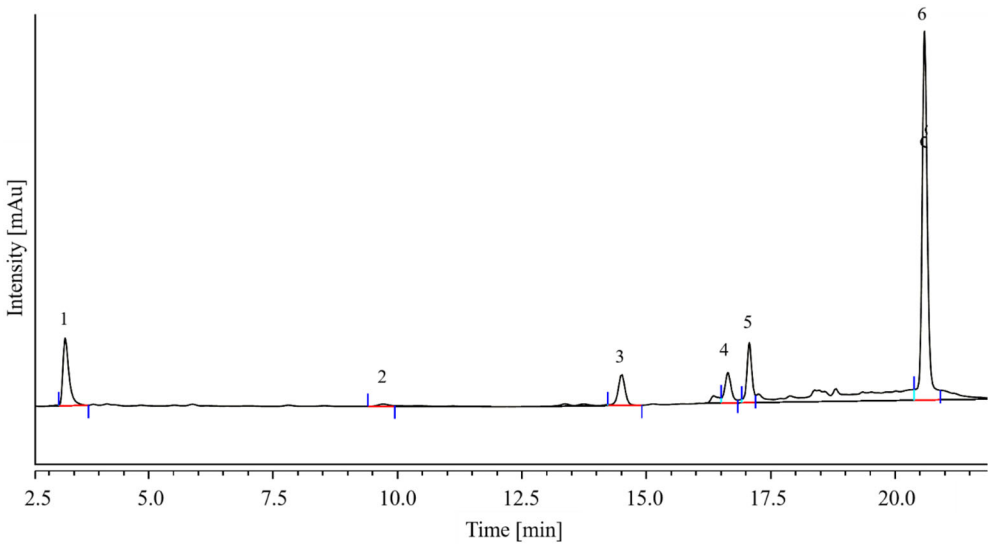

FIGURE 3 HPLC-DAD chromatogram of an aqueous coffee extract recorded at 272 nm. Analytes are numbered as follows: trigonelline (1), 5-hydroxymethylfurfural (HMF) (2), 3-caffeoylquinic acid (3-CQA, 3), 4-caffeoylquinic acid (4-CQA, 4), 5-caffeoylqunic acid (5-CQA, 5), caffeine (6).

TABLE 2 Validation results of the HPLC-DAD method considering linearity, limit of detection (LOD), and limit of quantitation (LOQ) for the eight analytes by regression of the determined concentration against the concentration actually present.

Analyte	R^2	LOD [g/kg]	LOQ [g/kg]
Trigonelline	0.9999	0.27	1.01
HMF	0.9997	0.38	1.41
3-CQA	0.9993	0.39	1.42
4-CQA	0.9890	1.26	3.86
5-CQA	0.9962	1.33	4.47
Caffeine	0.9988	0.82	2.89

Note: R^2: coefficient of determination, N = 8 in all equations.

trigonelline, CQA, and caffeine, similar concentrations were determined with both methods. By using ^{1}H-NMR calculated concentration ranges were 1.73–8.60 g/kg for trigonelline, 6.36–28.91 g/kg for CQA, and 10.93–14.65 g/kg for caffeine. For trigonelline, slightly lower concentrations were obtained by using HPLC-DAD (1.54–8.39 g/kg), whereas concentrations of CQA were slightly higher (8.95–32.23 g/kg). Levels of caffeine as determined by HPLC-DAD were almost the same (10.82–14.71 g/kg) as determined by ^{1}H-NMR.

Application of the ^{1}H-NMR method to coffee samples

The proposed ^{1}H-NMR method was applied to 603 arabica samples, and concentrations of lactic acid, acetic acid, formic acid, caffeine, CQA, NMP, trigonelline, and HMF were determined. Results are summarized in Box-Whiskers plots (Figure 5). Concentrations of lactic acid, formic acid, acetic acid, and caffeine showed a narrow scattering over all samples, whereas the amounts of HMF, NMP, trigonelline, and CQA displayed a broader distribution. These compounds are strongly influenced by the roasting process.[42] Because the coffee samples that were analyzed in this study were already roasted and showed different roasting degrees, the broader distribution of these analytes can easily be explained. Averaged over all coffee samples, concentrations of lactic acid, acetic acid, formic acid, caffeine, CQA, NMP, trigonelline, and HMF were 0.85 ± 0.13, 4.31 ± 0.50, 2.68 ± 0.47, 13.41 ± 2.19, 21.76 ± 5.19, 1.00 ± 0.22, 6.33 ± 1.16, and 0.37 ± 0.13 g/kg, respectively, based on the dry weight of the coffee samples. In 476 coffee samples, the concentration of NMP was below the LOQ of NMP. The same was true for HMF in 276 coffee samples, for acetic acid in 28 samples, for lactic acid in two samples, and for formic acid and trigonelline in one sample. The respective samples were not considered for the calculation of the average contents.

Rodrigues and Bragagnolo identified and quantitated among others CQA, trigonelline, caffeine, and HMF in coffee samples with a medium to dark roasting degree using HPLC-DAD.[43] However, quantities were related to the dry extract, which they determined by evaporation and differential weighing of aqueous coffee extracts.[43] Therefore, these data cannot be compared with our results. Gant et al. also quantitated caffeine, trigonelline, NMP, and HMF by using HPLC-DAD and obtained concentrations of 7.79 ± 0.09 g/kg for caffeine, 4.63 ± 0.07 g/kg for trigonelline, 0.29 ± 0.004 g/kg for NMP, and 0.29 ± 0.01 g/kg for HMF.[22] Thus, the amounts obtained by Gant et al. were slightly lower than the concentrations quantitated in this study. Galli et al. quantitated various organic acids using capillary electrophoresis and determined concentrations for lactic acid, acetic acid, and formic acid of 0.72 ± 0.2, 7.3 ± 0.3, and 2.7 ± 0.08 g/kg, similar to the concentrations quantitated here.[44]

Finally, Burton et al. quantitated trigonelline, caffeine, CQA, NMP, formic acid, and acetic acid in methanolic coffee extracts by ^{1}H-

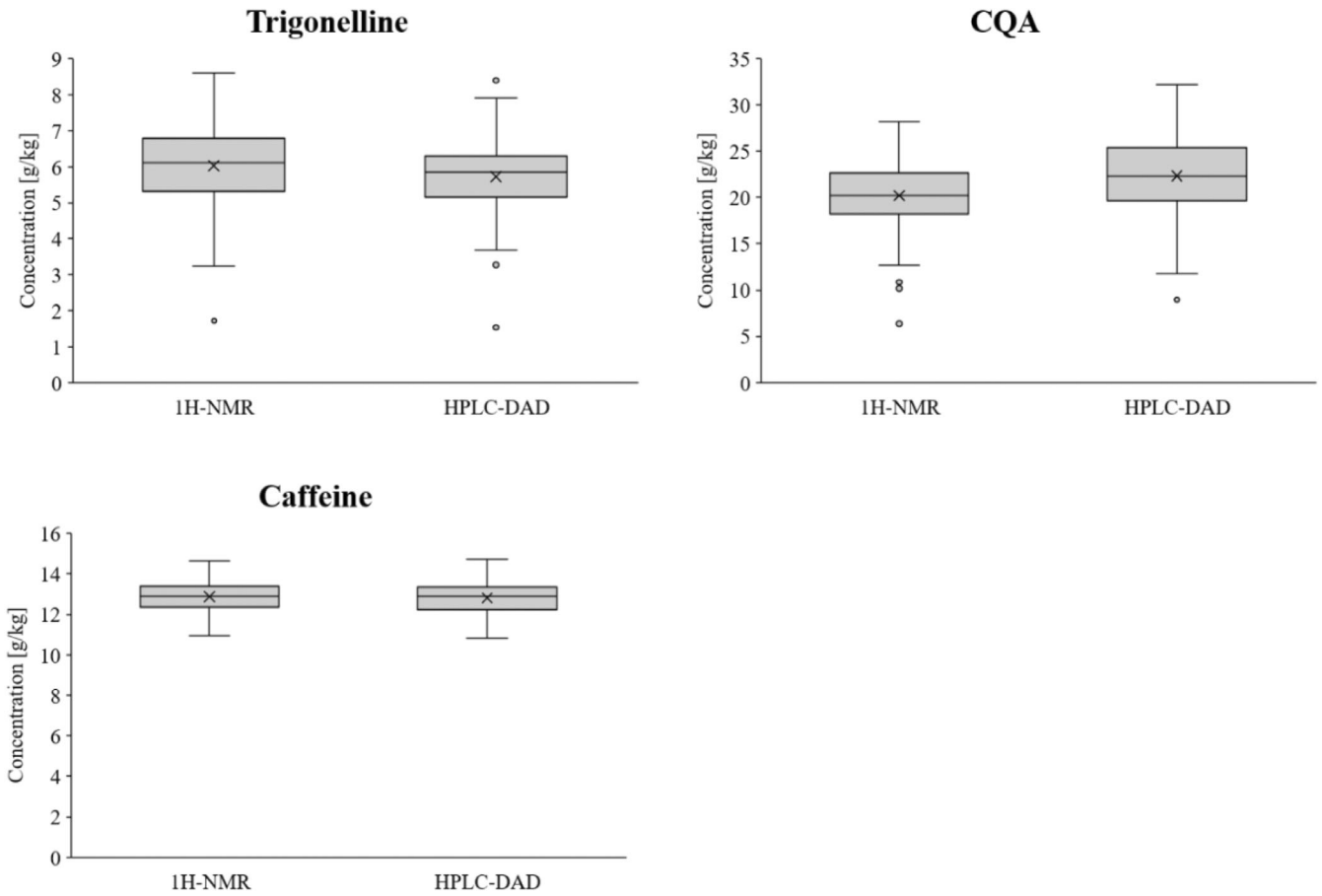

FIGURE 4 Box-Whiskers plots of the concentrations of trigonelline, caffeoylquinic acid isomers (CQA), and caffeine determined in 78 coffee samples by using the [1]H-NMR method and the HPLC-DAD method.

NMR.[20] The amounts of caffeine and NMP were similar to the concentrations obtained here. The amounts of trigonelline, CQA, acetic acid, and formic acid in the coffee samples analyzed by Burton et al. were, however, much lower compared with our results.[20] The differences could be due to the use of different solvents for extraction of the coffee samples.

Application of the [1]H-NMR method to coffee samples containing various defects

Coffee samples containing different defects were analyzed by using our [1]H-NMR method (Table S3). Defects can be categorized as follows: beans damaged during cultivation such as from pest or fungal infections, water or nutrient deficiencies (black beans, eaten beans, well eaten beans, quaker, malformed, unripe beans) and beans damaged during green coffee processing such as improper fermentation or incorrect drying (sour/brown beans, dark green beans, bleached beans, faded beans, pulper cuts, broken beans). Due to inadequate cleaning processes, residues of the coffee cherry, of the coffee plant or small twigs can get into the green coffee beans (husks, shells, parchment, pods, and twigs). Defects were classified after the Green Coffee Association of New York and, of the potential classes NY2-NY8, only the classes NY2, NY4, and NY6 were investigated in this study. No distinct differences were observed among the classification levels NY2, NY4, and NY6; thus, only the results of the class NY6 are shown (Table S3). Compared with the coffee without defects, slightly reduced concentrations of all analytes captured by the [1]H NMR method except formic acid were determined in the defect black beans, quaker, malformed, dark green beans, faded beans, beached beans, and pods. In particular, lower caffeine contents were quantitated for unripe beans, which involve the addition of coffee beans from unripe coffee cherries. This observation is in agreement with results obtained by Kidist et al., who also determined a lower caffeine content in coffee beans of unripe fruits.[45] Higher concentrations of HMF, NMP, formic acid, acetic acid, and lactic acid with lower concentrations of trigonelline and CQA were determined in coffee samples containing the defects broken beans and pulper cuts. These results may be explained by a stronger roasting of the damaged coffee beans and thus a higher amount of the roasting products. For coffee samples containing the defect twigs, higher concentrations of HMF and acetic acid were found, which could also be explained due to the roasting process. Changes in the

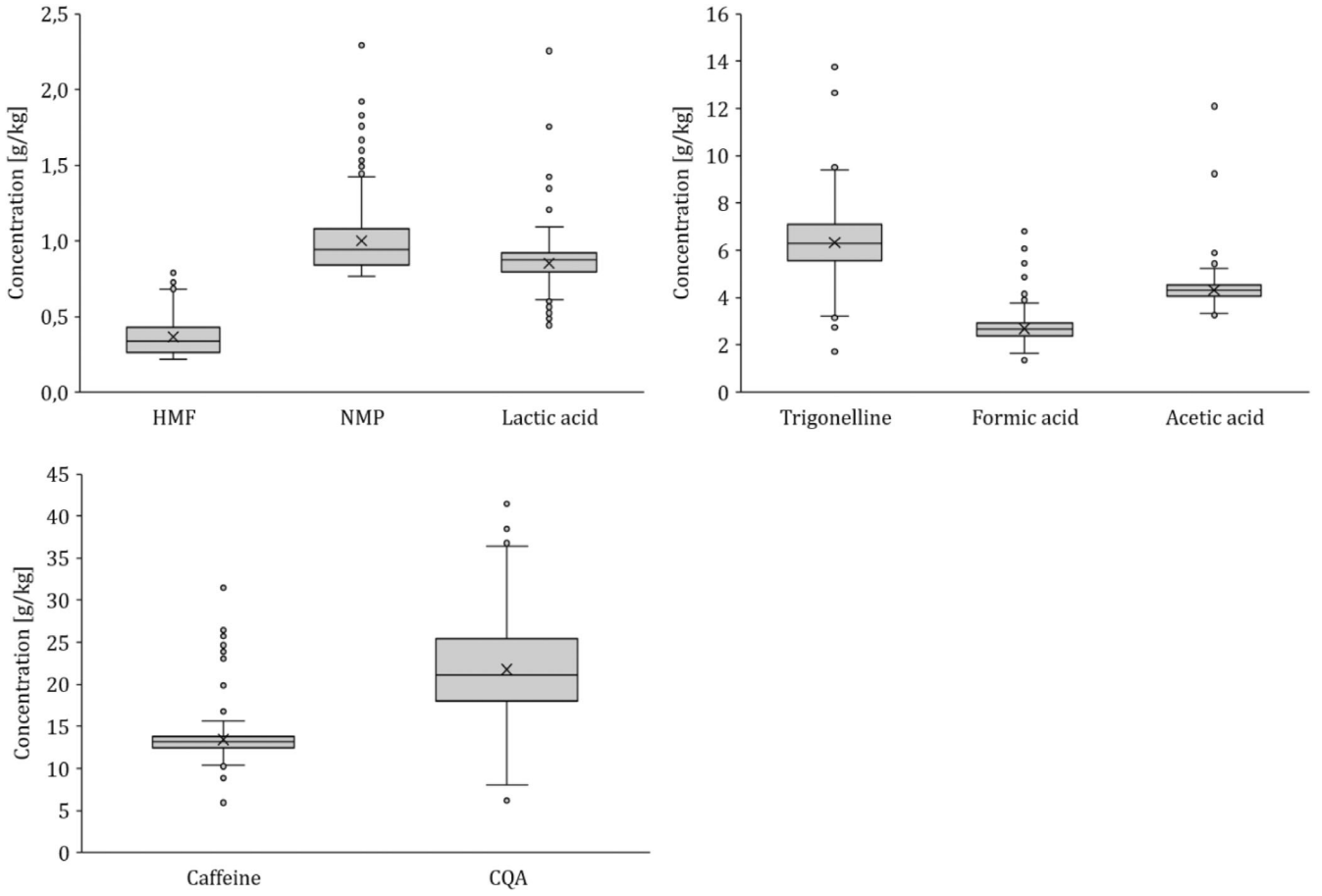

FIGURE 5 Box-Whiskers plots of the concentrations of 5-hydroxymethylfurfrual (HMF), N-methylpyridinium (NMP), lactic acid, trigonelline, formic acid, acetic acid, caffeine, and caffeoylquinic acid isomers (CQA) determined in coffee samples by using the ^{1}H-NMR method.

analyte concentrations when analyzing the defects shells and husks were minimal compared with the coffee sample without defect. Thus, potential addition of shells and husks would result in only minimal changes in the concentrations of the analytes studied here.

Overall, the defect coffee samples studied here show minimal changes in the concentrations of the analytes detected by our ^{1}H-NMR method. This is in agreement with Franca et al., who also suggested that there were no significant differences in the chemical composition of defect coffee samples and coffee samples without defects.[46]

CONCLUSION

A validated ^{1}H-NMR method was established, that allows a rapid (30 min) simultaneous and automatic quantitation of eight different quality markers in roasted coffee. These eight quality markers include organic acids, bioactive compounds and heat-induced contaminants of which some have an effect on the coffee aroma and on human health. A comparison of the ^{1}H-NMR method with an additionally developed and validated HPLC-DAD method showed similar concentrations of caffeine, CQA and trigonelline. Also, applying the HPLC-based German reference method for the determination of caffeine in coffee and coffee products resulted in similar concentrations of caffeine.

The ^{1}H-NMR method was successfully applied to 603 Coffea arabica coffee samples and to coffee samples containing various defects. Thus, the method is suitable for a quick, accurate and precise quality evaluation.

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CONFLICT OF INTEREST STATEMENT

In this work, there are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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