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Quantitative determination of caffeine, formic acid, trigonelline and 5-(hydroxymethyl)furfural in soluble coffees by ^{1}H NMR spectrometry

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ABSTRACT

A quantitative method for the determination of caffeine, formic acid, trigonelline and 5-(hydroxymethyl)furfural (5-HMF) in soluble coffees by applying the proton nuclear magnetic resonance technique (^{1}H NMR) is proposed. Each of these compounds records a singlet signal at the 7.6–9.5 ppm interval of the spectrum, and its area is used to determine the concentration. 3-(Trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid is added in an exact known concentration as a reference for δ = 0.00 ppm and as an internal standard. The method is applied to commercial soluble coffees and satisfactorily compared with results obtained by standard methods. The limits of detection and the coefficients of variation (N = 10) are, respectively, 1.32 mg/g of solid product and 4.2% for caffeine, 0.45 mg/g and 2.6% for formic acid, 0.58 mg/g and 2.4% for trigonelline, and 0.30 mg/g and 7.3% for 5-HMF. The described method is direct and no previous derivatization is needed.

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1. Introduction

Coffee is a widely consumed stimulant beverage prepared from roasted seeds, commonly called coffee beans, from the coffee plant. It has become a universal and almost indispensable beverage in modern life. The International Coffee Organization defined “green coffee” as “all coffee in the naked bean form before roasting”, “roasted coffee” as “green coffee roasted to any degree”, and “soluble coffee” as “dried water-soluble solids derived from roasted coffee” [1]. Once ripe, coffee seeds are picked and dried. The seeds are then roasted, undergoing several physical and chemical changes, and they are then ground and brewed to create coffee. Coffee can be prepared and presented in a variety of methods [2].

Caffeine is the most characteristic alkaloid compound present in coffee, because of its stimulant properties on the central nervous system. In fact, because of the popularity of coffee and other beverages that contain it like tea, soft drinks and energy drinks, caffeine is the world’s most widely consumed psychoactive substance. Trigonelline is another alkaloid present in coffee. Determination of caffeine and trigonelline levels in coffee is very important for the coffee industry, since they have a great effect on the final quality of the coffee products. Both compounds are involved in coffee bitterness and trigonelline has been associated with flavor formation and aroma production during coffee roasting [3,4].

Formic acid is the first and strongest of the unsubstituted carboxylic acid series. It occurs naturally in a variety of fruits, vegetables and leaves and roots of plants, and it is also present in coffee. It is involved in the perceived acidity of coffee, as it is almost fully deprotonated at the pH ∼ 4.8 given by coffee (pK_a = 3.75) [5].

5-(Hydroxymethyl)furfural (5-HMF, 5-hydroxymethyl-2-furancarboxaldehyde) is an aldehyde-furan compound formed during the thermal decomposition of sugars and carbohydrates. It is recognized as an indicator of quality deterioration, as a result of excessive heating or storage, in a wide range of foods like jams, fruit products, vegetable products and honey. In coffee, it is formed during the roasting process. Like caffeine and trigonelline, it is involved in the bitter taste perception [6–8].

Several strategies can be found in the bibliography for the determination of these compounds. Almost all are based on high performance liquid chromatography (HPLC) using different detectors [8–10]. There can be also found, among others, high performance gel filtration methods for caffeine and trigonelline [11], gas chromatography for trigonelline [12] and formic acid [13] and spectrophotometric methods for caffeine [14] and formic acid [15].

The ^{1}H NMR spectroscopy is capable to offer, in a single spectrum, the signals of many different compounds. This fact supposes that, potentially, a high number of chemicals might be simultaneously determined. Moreover, it also offers advantages in terms

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of speed and simplicity of sample preparations [16]. ^{1}H NMR has been used, together with chemometrics, to classify coffees according to their different origins or grain species [17,18]. Bosco et al. [19] performed the assignment of signals to many major constituents present in coffee, including caffeine, formic acid and trigonelline. Our research group has developed a quantitative determination of formic acid in apple juices by using its signal on the ^{1}H NMR spectrum [20]. However, to our knowledge, no quantitative procedures have been proposed for the other three analytes with this technique in coffee. There is only a previous determination of formic acid in coffee by using ^{1}H NMR, but it needs previous precipitation, liofilization and extraction with ethyl ether [21].

In the present work, we describe a new method for the quantitative and simultaneous determination of caffeine, formic acid, trigonelline and 5-HMF in soluble coffees by measuring their signals in the ^{1}H NMR spectrum at the 7.5–10.0 ppm interval. It is a fast and direct method, with no need of any previous derivatization or treatment, and requires 20 min to be performed.

2. Experimental

All chemicals used are of an analytical reagent grade, Aldrich, Sigma or Merck. Solutions are prepared with twice-distilled water (from this point on "water").

2.1. Preparing the TSP–D_2O solution

Exactly 0.0500 g of 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid sodium salt (TSP) are dissolved in 10 mL of water. 300 μL of this solution and 7 mL of D_2O are transferred to a 10 mL volumetric flask and the solution is made up to 10 mL with water. The final concentrations are 0.150 g/L of TSP and 70% (v/v) D_2O.

2.2. Recording of ^{1}H NMR spectra: general procedure

600 μL of the calibration standard or the coffee sample are placed in a 5 mm outer diameter NMR tube and 100 μL of the TSP–D_2O solution are added. The final concentrations are TSP 21.43 ± 0.14 mg/L and D_2O 10% (v/v). D_2O serves as the field frequency lock and all the spectra are referenced to the signal from TSP at $\delta = 0.00$ ppm. TSP is added in an exact known concentration as an internal standard.

500 MHz ^{1}H NMR spectra are recorded at a temperature of 30 °C using a Bruker DRX-500 spectrometer. 128 scans of 32 K data points are acquired with a spectral width of 8012 Hz (16 ppm), acquisition time of 4.0 s, recycle delay of 5.0 s, flip angle of 90° and constant gain of 28.5, requiring about 20 min per sample. Solvent suppression is achieved using the Watergate pulse sequence [22].

Preliminary data processing is carried out with Bruker software, TOPSPIN 1.3. The Free Induction Decay signals are Fourier transformed (1.0 Hz line broadening) and the spectra are phased and the baseline corrected. The resulting spectra are aligned by right or left shifting as necessary, using the TSP signal as a reference. Data analysis is achieved with MestReC 4.9.9.9 software package [23].

2.3. Determination of the longitudinal relaxation time, T_1

A solution is prepared by directly adding ~2.5 mg of each caffeine, trigonelline, 5-HMF and TSP in a NMR tube and dissolving them in ~700 μL of D_2O. To measure the T_1 of these compounds, the longitudinal relaxation delays of the selected protons are determined by the inversion recovery pulse sequence method, using T_1 cal Bruker program which fits the data to the exponential equation $I = I_0 + P\exp(-\tau/T_1)$, where I is the intensity of each proton resonance at inversion delay τ and I_0 at the equilibrium state, and P is a constant. Inversion delays used are 0.25, 0.50, 1.00, 2.00, 4.00, 8.00, 15.00, 30.00, and 60.00 s.

2.4. Calibration graphs

Caffeine 5000 mg/L and trigonelline 5000 mg/L stock solutions are prepared from the pure reagents. A formic acid stock solution of approximately 2000 mg/L is prepared from 98% commercial acid and the exact concentration is determined by titration with NaOH. A 5-(hydroxymethyl)furfural (5-HMF) stock solution of approximately 3000 mg/L is prepared from the pure reagent, a highly hygroscopic solid, and the exact concentration is determined by measuring the absorbance of the solution at 284 nm, where the molar absorptivity of the compound is 16,830 L/mol cm.

By diluting the stock solutions, 12 standards are prepared with concentrations in the range of 50–1600 mg/L for caffeine, 20–370 mg/L for formic acid, 20–640 mg/L for trigonelline and 12–320 mg/L for 5-HMF. The ^{1}H NMR spectrum of each standard is recorded following the general procedure. The calibration graphs are obtained by plotting the ratio between the peak areas of each analyte and the internal standard TSP against analyte concentration.

2.5. Preparing the samples

Commercial soluble coffees are used in this study: Baqué Natural (BAQUE), Eroski Tueste Natural (EROSKI), Fortaleza Natural (FORTA), Marcilla Crème Express Natural (MARCI), Nescafé Alta Rica (NES ALT), Nescafé Classic Natural (NES CLA), Nescafé Puro Colombia (NES COL), Nescafé Expresso (NES ESP), Nescafé Solo (NES SO), Spar Tueste Natural (SPAR). The abbreviations indicated in brackets are used later in the tables.

2 g of soluble coffee are placed in a conical flask. About 35 mL of boiling water are added, the conical flask is covered with a watch glass and boiling is continued gently for 15 min with magnetic stirring. The solution is quickly cooled to room temperature with running water, transferred to a 50 mL volumetric flask and filled up with water. About 10 mL of the last solution are clarified by centrifugation (1800 × g, 5 min) and filtration through a 0.45 nm filter.

A fraction of the clarified solution (<2 mL) is immediately used for ^{1}H NMR spectra recording and determination of the four compounds. The remaining clarified solution is frozen until the determination of caffeine, trigonelline and 5-HMF by HPLC is fulfilled.

1 mL of the non-clarified solution is diluted to 10 mL and discoloured by adding ~0.3 g activated charcoal powder (or more if necessary) and stirring for 15 min in an ultrasounds bath. The discoloured solution is centrifuged (1800 × g, 5 min), filtered through a 0.45 nm filter and frozen until formic acid is determined enzymatically (activated charcoal does not give any detectable signal with the enzymatic method, as confirmed by a previous blank experiment performed with water).

2.6. Analysis of samples by ^{1}H NMR

The ^{1}H NMR spectra of the clarified sample solutions are recorded as per the general procedure. The spectra are carried out in duplicate. Caffeine, formic acid, trigonelline and 5-HMF are determined from the spectra by using the area of the selected signals and the calibration graphs.

2.7. Analysis of samples by HPLC

After thawing the clarified sample, caffeine, trigonelline and 5-HMF are determined by HPLC using a Waters Sun Fire™ C18

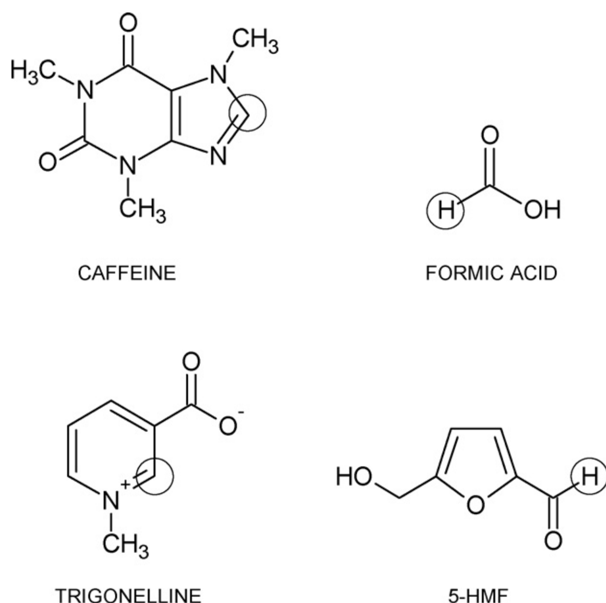
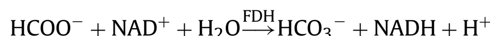


Fig. 1. Chemical formulae of the four compounds determined. The protons involved in the working ^{1}H NMR signals are indicated with circles.

column, 4.6 mm × 250 mm, 5 μm (Waters, Milford, MA, USA) and an Agilent 1100 high performance liquid chromatograph (Agilent, CA, USA). The operating conditions are as follows: isocratic elution, mobile phase 80% acetic acid (0.2%, pH 3.0) and 20% methanol, flow rate 2 mL/min, column temperature 40 °C and injected volume 20 μL. Column effluents are detected with a diode-array detector. Chromatograms are recorded at 273 nm for caffeine, 265 nm for trigonelline and 284 nm for 5-HMF, with reference at 800 nm where samples do not absorb. Analytes are quantified by the external standard method from peak areas. This HPLC method has been taken from Franca et al. [24], with some modifications.

2.8. Determination of formic acid in samples by an enzymatic method

After thawing the discoloured and clarified sample, a commercial enzymatic method [25] is used to determine formic acid. In this method, formic acid (as formate) is quantitatively oxidized to bicarbonate by nicotinamide-adenine dinucleotide (NAD^+) in the presence of formate dehydrogenase (FDH):



$$HCOO^- + NAD^+ + H_2O \xrightarrow{FDH} HCO_3^- + NADH + H^+$$

The amount of NADH formed, measured by its absorbance at 340 nm, is stoichiometric to the amount of formic acid.

3. Results and discussion

3.1. ^{1}H NMR spectra of coffee solutions

The chemical formulae of the four compounds determined are shown in Fig. 1. Fig. 2 displays the ^{1}H NMR spectrum of the commercial soluble Baqué coffee, with the TSP reference signal indicated at 0.00 ppm. Fig. 3 is an enlargement of the low field region of the spectrum (7.5–9.8 ppm). Similar spectra are obtained with other coffee solutions. References of caffeine, trigonelline and 5-HMF for the assignment of signals are prepared and analyzed in the same way as the samples are.

Caffeine gives three intense signals at 3.28, 3.45 and 3.88 ppm, produced by the three N-methyls, and the signal at 7.83 ppm, that corresponds to the aromatic proton. Formic acid gives a single signal at the 8.2–8.5 ppm interval, depending on the pH [20], generated by the non-carboxylic proton. At the pH ~ 4.8 given by dissolved coffee, the signal of formic acid occurs at 8.45 ppm.

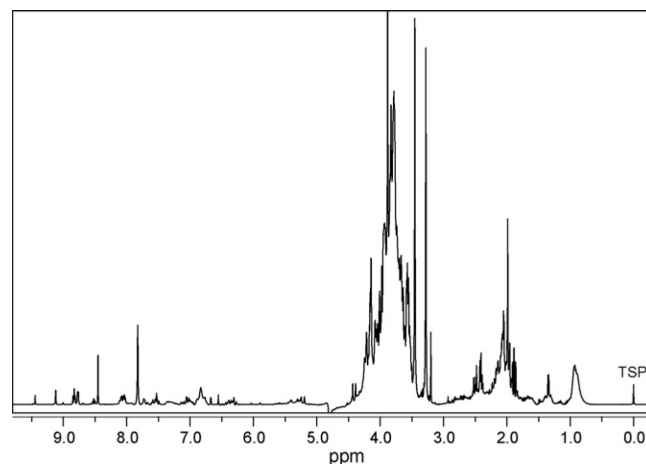


Fig. 2. ^{1}H NMR spectrum of Baqué soluble coffee. TSP signal is indicated.

Trigonelline produces four signals that correspond to the aromatic protons: a triplet at 8.09 ppm, two doublets at 8.82 and 8.84 ppm (overlapped in this spectrum) and a singlet at 9.12 ppm. N-methyl-pyridine, $C_5H_5N^+–CH_3$, is a cation with a similar structure to trigonelline and also present in coffee [19]. Its aromatic protons give a triplet signal at 8.04 ppm (proton in para), a triplet at 8.52 ppm (protons in meta) and a doublet at 8.77 ppm (protons in orto), which partially overlap with some trigonelline signals, particularly with the triplet at 8.09 ppm. The N-methyl groups of both trigonelline and N-methyl-pyridine give a single signal at 4.44 and 4.39 ppm, respectively.

5-HMF gives two doublet signals at 6.68 and 7.53 ppm, produced by the aromatic protons, and a singlet signal at 9.44 ppm, generated by the aldehydic proton. The methylene group gives a singlet signal at 4.70 ppm, not visible in this spectrum because the baseline is distorted in this area due to the Watergate pulse sequence used to suppress the water signal.

For quantitative determination purposes, the strongest non-overlapped signals of the analytes are chosen: 7.83 ppm for caffeine, 8.45 ppm for formic acid, 9.12 ppm for trigonelline and 9.44 ppm for 5-HMF. They are singlet signals located in the low field region of the spectrum (7.5–9.8 ppm, Fig. 3) which is, therefore, the working region for quantitative analysis. The signals have been indicated

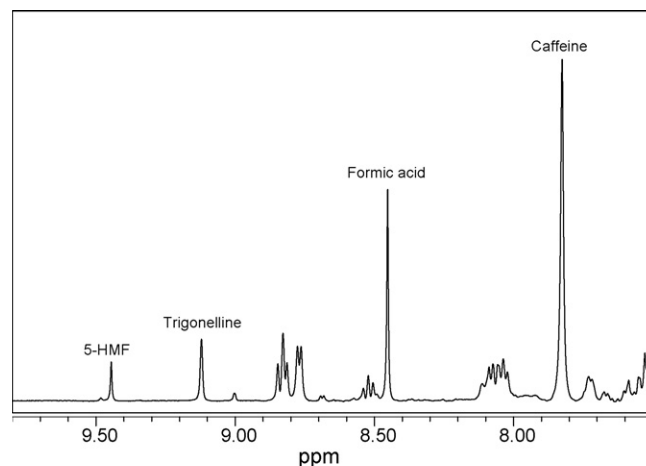


Fig. 3. Low field ^{1}H NMR spectrum of Baqué soluble coffee. Working signals of caffeine, formic acid, trigonelline and 5-HMF are indicated.

Table 1
Calibration data for the general equation $A/A_{TSP} = a \times C$ (mg/L) + b.

Analyte	$a \pm S_a$	$b \pm S_b$	R	$S_{y/x}$	LOD mg/L	LOD mg/g
Caffeine	$(4.832 \pm 0.054) \times 10^{-3}$	$(1.446 \pm 4.658) \times 10^{-2}$	0.9996	8.5655×10^{-2}	53	1.33
Formic acid	$(1.530 \pm 0.027) \times 10^{-2}$	$(3.968 \pm 5.653) \times 10^{-2}$	0.9989	9.3334×10^{-2}	18	0.45
Trigonelline	$(4.295 \pm 0.053) \times 10^{-3}$	$(5.811 \pm 18.16) \times 10^{-3}$	0.9995	3.3403×10^{-2}	23	0.58
5-HMF	$(4.854 \pm 0.062) \times 10^{-3}$	$(3.351 \pm 11.01) \times 10^{-3}$	0.9994	1.8674×10^{-2}	12	0.30

S_a: standard error for slope; S_b: standard error for intercept; $S_{y/x}$: standard error for regression line; LOD (mg/L): limit of detection from $3 \times S_{y/x} + b$; LOD (mg/g) = LOD (mg/L)/40 and $N = 9$ in all equations.

on the spectrum in Fig. 3 and the corresponding protons are highlighted with a circle on the formulae of Fig. 1.

The ^{1}H NMR acquisition conditions were optimized for formic acid in our previous work [20]. The critical factor in that case was the longitudinal relaxation time of the proton of formic acid, $T_1 = 11.651 \pm 0.002$ s. To obtain maximum relaxation of the molecule after each data acquisition and so that the registry times of the spectra not to be excessively long, the conditions selected were acquisition time 4 s, recycle delay time 5 s and number of scans 128. The T_1 value obtained here for the selected proton of caffeine is 5.6640 ± 0.0004 s, for trigonelline 5.7850 ± 0.0003 s, for 5-HMF 1.508 ± 0.041 s and for the internal standard TSP 3.769 ± 0.016 s. As formic acid has the longest T_1, the limiting compound is the acid itself, and so the same ^{1}H NMR acquisition conditions are used in this work, which requires a 20 min time lapse to record each spectrum.

3.2. Calibration equations and limits of detection

The calibration graphs are obtained by plotting the ratio between the peak areas of each analyte (A) and the internal standard TSP (A_{TSP}) against analyte concentration (C, mg/L). The general equation is $A/A_{TSP} = a \times C$ (mg/L) + b. The number of experimental points, N is 9 in all cases. As can be deduced from the sample preparation procedure, C (mg/L)/40 = C (mg/g of solid product). By processing the experimental data with the SPSS 14.0 statistical software package for Windows [26], the calibration equations listed in Table 1 are obtained. The high correlation coefficients obtained indicate a good linearity response within the concentration range studied. This was confirmed by the t-test [27], which gives high t values in all cases (>50) with a significance lower than 0.01.

3.3. Precision

The precision of the method is verified by a repeatability test. Following the general procedure, we obtain the ^{1}H NMR spectra of many samples prepared with Nescafé Classic soluble coffee and then, using the calibration equations, we calculate the concentration of the four analytes in each sample. For 10 measurements performed throughout 5 days the coefficients of variation obtained are 4.2% for caffeine, 2.6% for formic acid, 2.4% for trigonelline and 7.3% for 5-HMF.

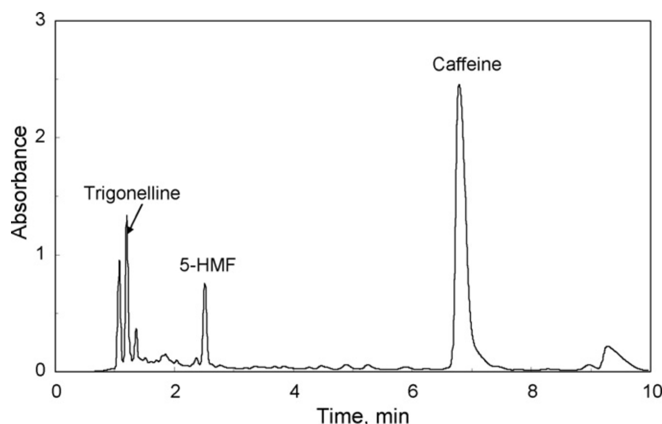


Fig. 4. Chromatogram of Fortaleza soluble coffee at 273 nm.

3.4. Application to real samples

To validate the ^{1}H NMR procedure described, we apply it to determine the four analytes in commercial soluble coffees. For comparison purposes, caffeine, trigonelline and 5-HMF are also determined by a HPLC standard method and formic acid with a commercial enzymatic method, using the procedures indicated in the experimental section. The chromatogram obtained at 273 nm for Fortaleza coffee is presented in Fig. 4. Very similar chromatograms are obtained with the other coffees. At 265 and 284 nm the chromatogram is the same but with more intense peaks for trigonelline and 5-HMF, respectively. We have to remark that the enzymatic method for formic acid failed when we applied it to apple juices [20] because of matrix effects. However, it works perfectly with discoloured and clarified coffee samples.

The results obtained are summarized in Table 2 (precision is in the same range of values reported in the previous section). To

Table 2
Determination of analytes in real samples by ^{1}H NMR and by HPLC or enzymatic standard methods.

Coffee sample	Caffeine (mg/g)		Trigonelline (mg/g)		5-HMF (mg/g)		Formic acid (mg/g)	
	^{1}H NMR	HPLC	^{1}H NMR	HPLC	^{1}H NMR	HPLC	^{1}H NMR	Enzymatic
BAQUE	37.69	38.84	6.06	6.77	2.37	2.38	4.07	4.11
EROSKI	38.88	42.55	5.17	6.21	1.69	1.79	4.20	4.55
FORTA	31.44	34.23	14.37	13.03	1.89	1.88	6.46	6.16
MARCI	35.86	36.11	6.33	6.87	0.62	0.95	4.40	4.25
NES ALT	21.64	20.96	8.63	8.91	2.92	2.65	7.30	6.45
NES CLA	33.61	31.69	5.56	6.34	1.59	1.63	4.74	4.66
NES COL	26.69	22.26	17.63[a]	14.84	6.18	5.13	6.70	6.86
NES ESP	24.30	23.97	13.47	11.92	2.18	2.15	7.23	6.44
NES SO	29.26	29.24	9.43	9.53	2.76	2.69	6.82	6.20
SPAR	35.80	39.25	8.63	8.47	1.61	1.68	5.86	5.66

[a] Value out of calibration range; it was obtained by diluting the sample twice and multiplying the NMR signal by 2.

Table 3
Paired t-test for results of Table 2.

Analyte	t	P
Caffeine	0.49	0.63
Formic acid	1.93	0.09
Trigonelline	0.60	0.56
5-HMF	0.77	0.46

Degrees of freedom = 9 and critical t = 2.26.

compare the results obtained by ^{1}H NMR with those obtained with the reference methods, the paired t-test is used [27]. Results are shown in Table 3. The experimental t value is under the critical t value and $P > 0.05$, indicating good agreement between ^{1}H NMR and reference methods.

These results prove that the method proposed is valid for the direct, simultaneous and quick determination of caffeine, formic acid, trigonelline and 5-HMF in soluble coffees. They also mean an extension of our method to determinate formic acid in apple juices by ^{1}H NMR to other food samples with greater contents of this analyte.

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