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Interaction mechanisms between caffeine and polyphenols in infusions of *Camellia sinensis* leaves

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ABSTRACT

Black tea infusions of *Camellia sinensis* leaves were studied for the influence of water composition, especially calcium content, on the amount of extracted organic matter and on the interactions between caffeine and polyphenols. The higher the calcium content, the lower the extraction of caffeine and polyphenols in acidic media. In alkaline media, besides the calcium effect, polyphenols are oxidized. Caffeine NMR chemical shifts varied depending on the water used showing modified interactions. Using model solutions, polyphenols seem to be responsible for these changes in the case of ultra pure water, but in the case of alkaline solutions, the data in model solutions are different from tea infusions implying that other compounds should interact. Moreover, epigallocatechin gallate (EGCg) and epigallocatechin are the polyphenols interacting most strongly with caffeine in infusions and not EGCg and epicatechin gallate as thought before.

1. Introduction

Catechin (C), epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg) and (−)-epigallocatechin gallate (EGCg) (Fig. 1) are the main phenolic flavan-3-ols constituting leaves from *Camellia sinensis* (Arce, Rios, & Valcarcel, 1998). Those compounds are thought to be beneficial for health due to their antioxidant, antimutagenic and anticarcinogenic properties (Almajano, Carbo, Lopez Gimenez, & Gordon, 2008; Arce et al., 1998; Trevisanato & Kim, 2000). Another active component present in tea leaves is caffeine (CAF; Fig. 1) (Trevisanato & Kim, 2000).

Tea cream is a precipitate formed as tea cools and is assumed to be due to complexation between CAF and polyphenols (Collier, Mallows, & Thomas, 1972; Roberts, 1963). Depending on the tea type, namely green (unfermented), oolong (partially fermented) or black (fully fermented), the nature of polyphenols interacting with CAF is different. CAF interacts with flavan-3-ols in the case of green and oolong teas or with oxidized polyphenols, *e.g.* theaflavins and thearubigins, in the case of black tea (Chao & Chiang, 1999; Liang, Lu, & Zhang, 2002). Those complexation phenomena seem to take place thanks to the A-ring for C, EC, EGC, to the galloyl group for ECg and EGCg (Fig. 1), and to the benzotropolone cycle for theaflavins and thearubigins (Charlton et al., 2000; Hayashi, Ujihara, & Kohata, 2004; Martin et al., 1986; Maruyama, Suzuki, Sakata, Yagi, & Ina, 1991). This complexation by stacking was also proposed by Baxter, Williamson, Lilley, and Haslam (1996) with the methyl gallate simple model in interaction with CAF.

Tea cream formation is controlled by several parameters, such as leaf–water ratio, extraction temperature and pH of the infusions (Chao & Chiang, 1999), and is favoured by calcium addition (Jöbstl, Fairclough, Davies, & Williamson, 2005). Tea leaves can uptake part of the calcium present in water used to brew them, which modifies the leaf structure (Anderson, Hollins, & Bond, 1971; Mossion, Potin-Gautier, Delerue, Le Hécho, & Behra, 2008). This leads to a decrease in the extraction of aluminium, total dissolved organic

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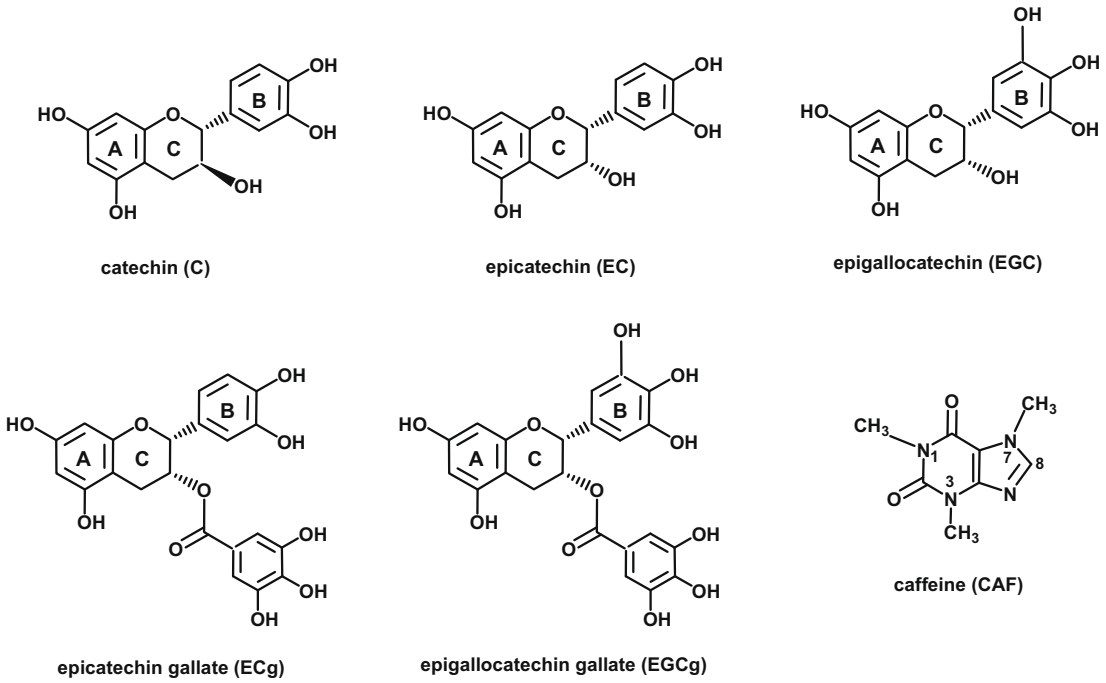

Fig. 1. Structures of catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECg), epigallocatechin gallate (EGCg) and caffeine (CAF).

carbon, total polyphenols and theaflavins (Mossion et al., 2008; Spiro & Price, 1987).

The originality of this work was to investigate the influence of the mineral composition of water, especially calcium content, on the interactions between CAF and polyphenols in tea infusions. Firstly, extracted organic matter was quantified using three complementary methods, *i.e.* total organic carbon (TOC)-meter, Folin–Ciocalteu method and NMR, whereas flavan-3-ols and alkaloids were assessed by HPLC–UV–MS, CAF being also quantified by NMR. The polyphenol–CAF interactions were then studied using zeta potential measurement and NMR. In order to improve the knowledge of interaction mechanisms, model aqueous solutions prepared by mixing CAF and polyphenols in concentrations as close as possible to those found in the real tea matrix were analysed by NMR. Finally the role of CAF, flavan-3-ols and calcium in tea cream formation, which is deleterious both for the consumer and for the producer, was investigated too.

2. Materials and methods

2.1. Chemicals, waters and tea

HPLC-grade acetonitrile and methanol were purchased from Sharlau (Berlin, Deutschland), gallic acid (GA), C, EC, EGC, ECg and EGCg from Extrasynthese (Genay, France), CAF, theobromine (TB), theophylline, $CaCl_2$, $CaSO_4$ and $CaCO_3$ salts from Fluka (Lyon, France), 3-(trimethylsilyl)-1-propane sulfonic acid (TMPS) from Acros Organics (Noisy le Grand, France) and sodium carbonate from Panreac (Lyon, France). A multi-element standard solution containing 100 mg/l of Ca, K, Mg, Na and also Al, As, Ba, Be, Bi, Cs, Ga, Ln, Li, Rb, Se, Sr was purchased from CPI-International (Amsterdam, The Netherlands).

Nitric acid (69%, purum reagent) and decarbonated sodium hydroxide (Fluka, HPIC grade) were used for pH adjustments. Buffer solutions at pH 4.0 and 7.0 (Fisher Bioblock Scientific, Illkirch, France) were used to calibrate the pH-meter.

Seven different waters, the compositions of which are given in Table 1, were used to prepare infusions: ultra pure water (A) prepared using a Milli-Q system (Model Gradient A10, Millipore), two mineral waters: a weakly (B) and highly (C) mineralised, and four so called synthetic waters (B + $CaCl_2$L(Low), B + $CaCl_2$H(High), B + $CaSO_4$, B + $CaCO_3$) prepared from water B to reach a total concentration of calcium close to water C calcium content before heating (analysed at $[Ca^{2+}] = 200$ mg/l). Waters A, B, B + $CaCl_2$L, B + $CaCl_2$H and B + $CaSO_4$ were considered to be unbuffered with respect to pH in contrast to buffered waters C and B + $CaCO_3$.

A Darjeeling loose tea with whole leaves from Namring area named M8 was purchased from a French tradesman. This tea has undergone an orthodox process, which consists in withering and rolling fresh tea leaves without broking them. Then, polyphenols are oxidized by enzymes during a phase of fermentation (Goodsall, Hodges, Jones, Mawson, & Stabler, 1999).

2.2. Anion and cation quantification

Anions in water were measured in triplicate at 20 °C using a Dionex ICS-2000 system. Samples were passed through an AG18 guard and AS18 (functional group: alkanol quaternary ammonium ion) analytical columns (Dionex) with a flow rate of 1.0 ml/min and a NaOH mobile phase. The gradient employed was as follows: 0–1 min, 23 mmol/l; 1–8 min, linear gradient 23–40 mmol/l; 8–12 min, linear gradient 40–52 mmol/l; 12–13 min, linear gradient 52–23 mmol/l; 13–21 min, 23 mmol/l. The injection volume was 25 µl. The suppressor current was set at 129 mA.

Ca and Mg concentrations were determined using an ICP-AES (inductively coupled plasma-atomic emission spectrophotometry) instrument (Panorama, Jobin–Yvon). The following conditions established after optimisation of the instrument parameters were: Rf power 1000 W; plasma gas flow rate 15 l/min; nebuliser gas flow rate 0.1 l/min; nebulizer cross-flow type; sample uptake 1.0 ml/min. K and Na were measured by atomic emission spectrophotometry (410, Corning). Wavelengths used were 589 nm for Na and 767 nm for K. The analyses were carried out in triplicate.

Table 1
Composition of waters after their heating at 95 °C.

Waters	pH	Cations (mg/l)				Anions (mg/l)			
		K^+	Na+	Mg^{2+}	Ca^{2+}	SO_4^{2-}	NO_3^-	HCO_3^-	Cl^-
A	5.7–6.5	n.d.[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B	7.1	2.2 ± 0.5	0.5 ± 0.1	0.6 ± 0.1	1.4 ± 0.1	2.7 ± 0.02	2.7 ± 0.03	2.3 ± 0.1	2.9 ± 0.1
C	8.0	7.7 ± 0.5	47 ± 1	2.5 ± 0.1	151 ± 8	359 ± 3	5.5 ± 0.4	156 ± 1	11.0 ± 0.2
B + $CaCl_2L$[b]	6.8	0.4 ± 0.1	2.3 ± 0.4	0.80 ± 0.01	168 ± 9	2.5 ± 0.2	2.8 ± 0.1	2.5 ± 0.1	254 ± 3
B + $CaCl_2H$[c]	6.9	0.3 ± 0.1	2.1 ± 0.4	0.80 ± 0.01	197 ± 3	2.6 ± 0.2	2.9 ± 0.1	3.5 ± 0.1	358 ± 2
B + $CaSO_4$[d]	6.7	0.4 ± 0.1	3.1 ± 0.4	0.81 ± 0.01	216 ± 1	474 ± 4	3.2 ± 0.1	2.4 ± 0.1	3.07 ± 0.04
B + $CaCO_3$[e]	8.0	0.6 ± 0.1	2.9 ± 0.4	0.80 ± 0.01	225 ± 7[f]	32.5 ± 0.3	2.9 ± 0.1	3.5 ± 0.1	3.42 ± 0.07

[a] n.d.: not detected.
[b] Theoretical $[Ca^{2+}]$ = 144 mg/l, $[Cl^-]$ = 256 mg/l.
[c] Theoretical $[Ca^{2+}]$ = 200 mg/l, $[Cl^-]$ = 355 mg/l.
[d] Theoretical $[Ca^{2+}]$ = 193 mg/l, $[SO_4^{2-}]$ = 463 mg/l.
[e] Theoretical $[Ca^{2+}]$ = 193 mg/l.
[f] Total calcium content after acid digestion (Mossion et al., 2008); $[Ca^{2+}]_{dissolved}$ = 15.1 ± 0.2 mg/l.

2.3. Preparation of tea infusions and pH measurements

Tea infusions were prepared according to the advice given by the tradesman: 150 g of water at 95 °C were added to 3 g of leaves in a tea pot. After 3 min, the infusions were filtered through a sieve (Nylon) for removing large particles and leaves, and placed in glass flasks for storage. The infusions were named according to the water used to prepare them followed by the suffix M8 (e.g. AM8 represents the tea infusion prepared with the ultra pure water A).

For HPLC and dissolved organic carbon (DOC) measurements, infusions were filtered through 0.45 μm cellulose acetate filters (Nalgene). The so called blank water followed the same procedure without tea leaves. pH measurements were carried out after cooling at ambient temperature, using a 330 pH-meter (WTW) with a combined Sentix 50 electrode (WTW).

2.4. Preparation of model solutions for NMR

CAF model solutions were prepared in waters A, B, C, B + $CaCl_2L$ and B + $CaCl_2H$ at two concentrations, 300 mg/l (CAF_{300}) or 600 mg/l (CAF_{600}), and pH was modified between 5 and 8 by adding nitric acid or sodium hydroxide to study its effect on CAF (chemical shifts)δ.

Polyphenol–CAF interactions were studied in model solutions with concentrations identical to those measured in the different tea infusions. In a first series of experiments, each polyphenol was weighed in an NMR tube and a solution of CAF prepared in water A or C was then added. In a second set of experiments, concentrated solutions of polyphenols prepared in water A or C were mixed before addition to a CAF solution prepared in water A or C.

2.5. Dissolved organic carbon (DOC) and total polyphenol content (TPC) measurements

DOC was measured using a TOC analyzer (TOC-5000A; Shimadzu) after filtration by difference between total and inorganic carbon values. Analyses were performed in triplicate.

TPC was determined using the Folin–Ciocalteu method (Montreau, 1972). In a graduated flask, 0.5 ml tea extract, 5 ml Folin–Ciocalteu reagent (Panreac) and 10 ml sodium carbonate (20%) were added and the volume adjusted to 100 ml with ultra pure water. The solutions were allowed to stand at 70 °C for 10 min before cooling and absorbance measurements at 700 nm on a Hewlett Packard 8452A spectrophotometer. TPC was expressed in grams equivalent to the standard used, *i.e.* GA, per litre of aqueous solution, noted g eq GA/l. Analyses were performed in triplicate.

In order to compare the results of DOC and TPC, the latter ones were expressed as mol C/l according to the following equation:

$$x = \frac{[TPC]}{M} \times 7$$

with x is the polyphenol content expressed in mol C/l, [TPC] is the polyphenol content expressed in g eq GA/l, M is 170.12 g/mol, the molecular weight of GA ($C_7H_6O_5$), 7 is the corrective factor to take into account the number of carbon atoms in one GA molecule.

2.6. HPLC analysis of flavan-3-ols and alkaloids

The HPLC system was a Dionex, including a P680 pump with a ASI-100 autosampler, coupled with a UVD 170/340U photodiode array detector and a mass spectrometer Surveyor MSQ (Thermo Electron Corporation). The separation was performed on an Omnisepher® (Varian) C18 stainless steel column (100 × 3.0 mm i.d.; particle size, 3 μm) at 30 °C. The flow rate was 0.4 ml/min. The gradient elution used two solvents, S_a (0.5% acetic acid in water) and S_b (0.5% acetic acid in 60/40 (v/v, %) methanol and acetonitrile), and was as follows: 10 min of equilibration at 95% S_a, then from 0 to 25 min, 95–70% S_a, from 25 to 38 min, 70–0% S_a, from 38 to 48 min, 0% S_a. The injection volume was 10 μl. The scan measurements, using positive electrospray mode, were performed with the following settings: heater temperature of nitrogen gas, 450 °C; capillary voltage, 3 kV; sheath gas, 45 psi (3.1026 kPa); scan range, 173–1972 m/z. Detection was done in single ion monitoring mode at m/z 139, 195, 291, 459 and 443. UV calibration curves for GA, C, EC, ECg, EGC, EGCg, CAF, TB and theophylline were recorded daily at 273 nm using standard solutions.

2.7. Zeta potential measurements

Zeta potential measurements were performed on a Malvern Zetasizer 4. This apparatus includes a microprocessor that first measures the electrophoretic mobility of colloidal particles dispersed in aqueous solutions, and then automatically calculates the zeta potential (in mV) using the Smoluchowski equation (Hunter, 1981). The accuracy of the Zetasizer was determined using zeta potential transfer standard (DTS1230, Malvern). All measurements were conducted in triplicate on unfiltered samples at 25 °C. Measurements were also conducted with two pH adjustment paths using a DL 21 titrator (Mettler). The first path started at the pH of the infusion and stepwise increased to pH 12 by adding sodium hydroxide. The second path also started at the pH of the infusion and decreased to pH 1 by adding nitric acid. Six measurements were performed at each step. For those measurements,

water B containing 400 mg/l Ca^{2+}, called B + $CaCl_2$400, was used to better show the role of calcium in the value of particle charges.

2.8. ^{1}H NMR spectroscopy

Immediately after preparation, black tea infusions (n = 3 for each infusion) were put in 5 mm diameter NMR tubes and their ^{1}H NMR spectra (n = 3 for each infusion) recorded at 25 °C on a Bruker AVANCE 500 spectrometer equipped with a 5 mm triple resonance proton cryoprobe (TCI) with z-axis gradients. TMPS was used as an internal reference.

1D ^{1}H NMR spectra with an excitation sculpting (ES) sequence for water signal suppression were acquired in the following conditions: 16 scans, 32 K data points, acquisition time 2.72 s, 90° flip angle, relaxation delay 1 s, 12 ppm spectral window.

For the quantitative NMR analysis, 500 µl of each infusion were lyophilised immediately after preparation. The pellets were redissolved in 500 µl of D_2O and analysed. 1D ^{1}H NMR spectra were recorded in the following conditions: 256 scans, 32 K data points, acquisition time 2.72 s, 30° flip angle, relaxation delay 6 s, 12 ppm spectral window.

For kinetic measurements, 200 1D ^{1}H spectra of tea infusions with a classical water suppression sequence using selective irradiation were recorded for a total of 13 h (4 min for each experiment) in the following conditions: 32 scans, 32 K data points, acquisition time 2.72 s, 30° flip angle, relaxation delay 2 s, 12 ppm spectral window.

^{1}H–^{1}H DQF-COSY spectrum was recorded with 2042 real points in acquisition time and 128 points for the second dimension. Twelve scans were collected and 12 ppm were used for the spectral window with a relaxation delay of 1.5 s. Raw data were multiplied by a squared cosine window function and Fourier transformed to obtain a final matrix of 2048 × 256 before polynomial baseline correction in both dimensions.

All ^{1}H NMR data sets were processed and analysed using Bruker TOPSPIN software package.

For 2D ^{1}H Diffusion-Ordered SpectroscopY (DOSY) NMR experiment, a bipolar pulse pair stimulated echo sequence including ES scheme for water suppression (Balayssac, Delsuc, Gilard, Prigent, & Malet-Martino, 2009) with spoiler gradients of −7.92 G/cm, an eddy current delay of 10 ms, 100 ms for diffusion time, 3.5 ms for pulse field gradient length and 3 ms for the gradient recovery delay was applied. Forty experiments were acquired in the diffusion dimension with intensity linearly sampled from 5% to 95% and gradient system calibrated to 46.25 G/cm. The acquisition time was 0.68 s with a spectral window of 12 ppm. Thirty-two scans and 1s as relaxation delay were used. The DOSY experiments were processed and analysed using NPK (Tramesel, Catherinot, & Delsuc, 2007) and NMRnotebook™ softwares, respectively.

3. Results and discussion

3.1. Water composition effect on the pH of infusions

The pH values are 5.4 for tea infusions prepared with waters A, B, B + $CaCl_2$L or H, B + $CaSO_4$, 7.3 with B + $CaCO_3$ and 7.6 with water C (Table 2). Compared to corresponding blank waters (Table 1), tea infusions are globally more acidic because the organic matter extracted contains carboxylic and phenolic groups (Erdemoglu & Gücer, 2005). The slight alkalinity of tea infusions prepared with waters C and B + $CaCO_3$ is due to their calco-carbonated system (Sigg, Behra, & Stumm, 2006).

3.2. ^{1}H NMR spectra of tea infusions

1D/2D ^{1}H NMR spectroscopy is an established technique to identify the main organic components of a mixture. In addition to the classical 1D and 2D NMR experiments, 2D DOSY ^{1}H NMR spectra were also recorded. The method is based on the difference in diffusion coefficient values between compounds of different molecular weights. The 1D version of this specific NMR spectroscopy is generally used for diffusion coefficient measurements (Zheng, Stait-Gardner, Anil Kumar, Torres, & Price, 2008). However, the introduction of a second dimension can simplify the analysis as the ^{1}H δ are observed on the direct dimension and the diffusion coefficients on the indirect dimension (Morris & Johnson, 1993). Fig. 2A illustrates the DOSY spectrum of the AM8 infusion. Signals were assigned according to the connectivities observed on the 2D DQF-COSY spectrum and the literature (Le Gall, Colquhoun, & Defernez, 2004). The separation along the diffusion axis enables to assign ^{1}H signals belonging to one organic compound. To confirm assignments, authentic standards of C, EC, ECg, EGC, and EGCg were added in AM8 and CM8 infusions and the spectra were recorded after spiking. The region of 1D ^{1}H NMR spectra between 5.4 and 8.1 ppm of AM8 and CM8 infusions is shown enlarged in Fig. 2B and C.

Black tea infusions contain theaflavins and thearubigins. Those compounds result from polyphenol oxidation and contribute to organoleptic properties, for example tea colour (Charlton et al., 2000). The signals of the benzotropolone group characteristic of theaflavin (Davis, Cai, & Davies, 1995) were not detected in the seven black tea infusions analysed. Depending on manufacture pro-

Table 2
pH and organic matter measurements with three analytical techniques (dissolved organic carbon (DOC), total polyphenol content (TPC) and NMR) for the seven infusions analysed.

Infusion	pH	DOC[a,b]		TPC[a,c]		Ratio TPC (mol C/l)/DOC (mol C/l)	DOC–TPC (mol C/l)	R_1[a,d]	R_2[a,e]
		mg C/l	mol C/l	mg eq GA/l	mol C/l				
AM8	5.4	1780 ± 28	0.148 ± 0.002	1244 ± 32	0.051 ± 0.001	0.35 ± 0.01	0.097 ± 0.003	1.00 ± 0.07	1.00 ± 0.05
BM8	5.4	1335 ± 57	0.111 ± 0.005	1104 ± 54	0.045 ± 0.002	0.41 ± 0.04	0.066 ± 0.007	0.94 ± 0.08	0.89 ± 0.07
CM8	7.6	1249 ± 20	0.104 ± 0.002	708 ± 63	0.029 ± 0.003	0.28 ± 0.03	0.075 ± 0.005	0.61 ± 0.12	0.57 ± 0.11
B + $CaCl_2$LM8	5.4	1400 ± 100	0.117 ± 0.008	761 ± 50	0.031 ± 0.002	0.27 ± 0.04	0.08 ± 0.01	0.66 ± 0.11	0.61 ± 0.09
B + $CaCl_2$HM8	5.4	1164 ± 60	0.097 ± 0.005	657 ± 40	0.027 ± 0.002	0.28 ± 0.03	0.070 ± 0.007	0.56 ± 0.10	0.53 ± 0.08
B + $CaSO_4$M8	5.4	1380 ± 40	0.115 ± 0.003	723 ± 78	0.030 ± 0.003	0.26 ± 0.04	0.085 ± 0.006	0.64 ± 0.11	0.58 ± 0.13
B + $CaCO_3$M8	7.3	1706 ± 34	0.142 ± 0.003	1041 ± 32	0.043 ± 0.001	0.30 ± 0.02	0.099 ± 0.004	0.82 ± 0.16	0.84 ± 0.05

[a] The infusions were prepared in triplicate and each replicate was analysed three times.
[b] DOC: dissolved organic carbon.
[c] TPC: total polyphenol content.
[d] $R_1 = \frac{[NMR_{int}]_X}{[NMR_{int}]_A}$ with $[NMR_{int}]_X$ is the value of the integration of the catechin signals in the 5.5–7.2 ppm region in the different tea infusions and $[NMR_{int}]_A$ is the value of the integration of the same aromatic region in the tea infusion prepared with water A (AM8).
[e] $R_2 = \frac{[TPC]_X}{[TPC]_A}$ with $[TPC]_X$ is the TPC content in the different tea infusions and $[TPC]_A$ is the TPC content in the tea infusion prepared with water A (AM8).

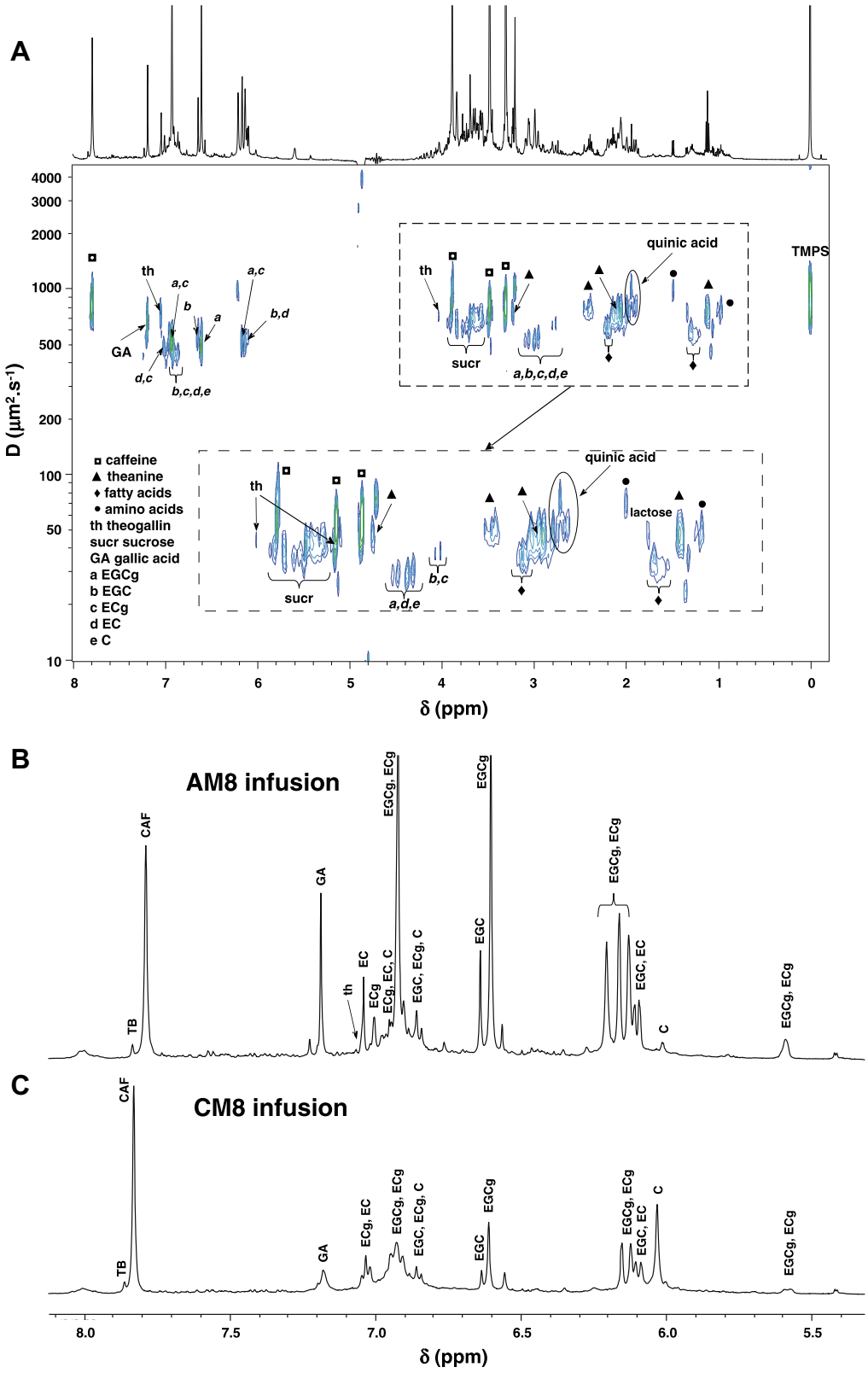

Fig. 2. 2D DOSY-ES ^{1}H NMR spectrum of the black tea infusion AM8 (A) and 1D ^{1}H NMR spectra of AM8 (B) and CM8 (C) infusions showing polyphenol signal assignments. Spectra were recorded at 25 °C with 10% D_2O. TB: theobromine, CAF: caffeine, GA: gallic acid, EC: epicatechin, ECg: epicatechin gallate, C: catechin, EGCg: epigallocatechin gallate, EGC: epigallocatechin.

cess and geographical origin, theaflavins and thearubigins represent 0.3–2% and 4–20% of dry matter of tea infusions, respectively (Haslam, 2003). As the leaves used in the present study underwent orthodox process that leads to the lowest level of oxidation, the contents in theaflavins and thearubigins should be too low to allow the detection of their ^{1}H NMR signals.

3.3. Organic matter extraction

Three methods were used for evaluating organic matter extraction, NMR, DOC and TPC measurements. The data are reported in Table 2.

^{1}H 1D NMR spectra show that the same organic tea components were extracted whatever the water used but with variable intensities. For example, the amount of extracted polyphenols decreases in the case of water C (Fig. 2C) with respect to water A (Fig. 2B), which is consistent with DOC and TPC measurements.

From data presented in Table 2, it is clear that calcium present in water reduces the extraction of organic matter, *e.g.* DOC and TPC values decrease from 1780 mg C/l and 1244 mg eq GA/l in water A containing no measurable Ca^{2+} to 1249 mg C/l and 708 mg eq GA/l in water C containing 151 mg Ca^{2+}/l. Moreover, the lowest extraction in the case of B + $CaCl_2$HM8 infusion, with 1164 mg C/l and 657 mg eq GA/l for DOC and TPC values, was obtained with one of the most calcium concentrated water ([Ca^{2+}] = 197 mg/l) (Table 1). As total polyphenols were determined without filtration, the decrease in the extraction of organic matter is not due to a possible loss at the filtration step. In the case of the infusion prepared with water B + $CaCO_3$, the extraction of organic matter is similar (1706 mg C/l and 1041 mg eq GA/l for DOC and TPC values) to that measured in the AM8 infusion (1780 mg C/l and 1244 mg eq GA/l for DOC and TPC values) prepared with the ultra pure water A, despite the differences in Ca^{2+} contents (0 and 225 mg/l). This might be due to the fact that calcium could not be uptaken by leaves since it was present in the infusion B + $CaCO_3$M8 as colloids due to the oversaturation of calcite in the solution after heating (Sigg et al., 2006).

The ratios of NMR integrations (R_1) are in agreement with those of TPC results (R_2) for all infusions, which shows that ^{1}H NMR allows a correct quantification of polyphenols. Polyphenols represent 26–41% of the total organic matter extracted (ratio TPC/DOC, Table 2). These changes might be induced by the variable extraction of other organic components in tea leaves such as proteins or polysaccharides, which is corroborated by the different values of [DOC–TPC] (Table 2) (Harbowy & Balentine, 1997).

3.4. Flavan-3-ol and alkaloid quantification

Six polyphenols (GA, C, EC, ECg, EGC, EGCg) and two alkaloids (CAF, TB) were quantified by HPLC–UV–MS (Table 3). In the different samples, theophylline was never detected. Only CAF concentration was determined by NMR because overlaps of polyphenol resonances (Fig. 2B and C) preclude their exact quantification. The CAF data are close when comparing both methods (Table 3). Furthermore, in our experimental conditions, the signal-to-noise ratio for TB is not sufficient for an accurate NMR quantification (Fig. 2B and C).

CAF extraction depends on water composition, since quantities decrease by ≈20% between waters A and C (from ≈450 to ≈360 mg/l). With respect to flavan-3-ols, the extraction of EGCg among the other polyphenols is the most affected, decreasing by a mean factor of ≈6 between AM8 and CM8 infusions (Table 3). Calcium dependence and polyphenol oxidation could explain the variability in extractions. When waters used to brew tea have an acidic or neutral pH, namely waters A, B, B + $CaCl_2$L and H and B + $CaSO_4$, both EGCg and CAF extractions are affected by water calcium content that increases from 0 to 216 mg/l. For example, EGCg and CAF amounts drop respectively by 33% and ≈10% in BM8 infusion and by ≈50% and ≈30% in B + $CaCl_2$HM8 infusion compared in both cases with AM8 infusion. With alkaline pH waters, *i.e.* C and B + $CaCO_3$ that have buffer capacity, the concentrations of polyphenols are often not reproducible between two experiments (*e.g.* 39 and 70 mg/l for GA in CM8, Table 3). According to Zhu, Zhang, Tsang, Huang, and Chen (1997), the polyphenol instability in alkaline medium which induces their oxidation during infusion preparation could explain this difference. The effect of the oxidation cannot be shown in TPC analyses since the Folin–Ciocalteu method is actually based on this oxidation in alkaline media.

3.5. Interactions between polyphenols and CAF as a function of water

Despite analytical difficulties due to the complexity of the real matrix, interactions between CAF and polyphenols were first studied by ^{1}H NMR on the seven black tea infusions just after brewing. Spectra analysis indicates that only CAF and polyphenol δ depend on the composition of the different waters used. For example, Fig. 3 illustrates the behaviour of the H_8 (7.80 ppm) resonance of CAF in the various infusions. A value of 21.0 ± 2.5 Hz (≈0.04 ppm) separates the signal in AM8 (right) and CM8 (left) infusions. However, no clear trend could be withdrawn from these data concerning the effect of type of water, pH, polyphenol and calcium contents on interactions between CAF and polyphenols.

Model solutions were thus prepared to independently test the influence of these parameters. Solutions containing 300 or 600 mg/l (CAF_{300} or CAF_{600}) of CAF were prepared in five waters (A, B, C, B + $CaCl_2$L and H), at various pH comprised between 5 and 8. The data presented in Fig. 3B and Table 4 show that the type of water, pH and calcium have a negligible effect on the δ of CAF protons for both solutions (*e.g.* variation of 0.45 Hz for N_1–Me and N_3–Me, 0.35 Hz for N_7–Me and 0.22 Hz for H_8 for the CAF_{300} solution). If CAF_{600} and CAF_{300} are now compared, a slight variation was observed for CAF methyl groups (1.6–3.2 Hz), but not for the H_8 proton (0.5 Hz, Table 4), which is due to a stacking effect of CAF molecules that affects the H_8 δ less than the CH_3 δ (Charlton et al., 2000). On the other hand, the highest variations of δ are observed for the H_8 in tea infusions with a minimum change (30 Hz) in the CM8 infusion and a maximum (51 Hz) in AM8 (Table 4).

Table 3
Quantification of flavan-3-ols and theobromine in tea infusions by HPLC–UV–MS and CAF by HPLC–UV–MS and ^{1}H NMR.

[Ca^{2+}] in blank waters (mg/l)	Infusions	Concentration (mg/l) in infusion[a]							CAF	
		GA	C	EC	EGC	ECg	EGCg	TB	HPLC	^{1}H NMR
n.d.	AM8	89	29	44	80	176	547	27	416	480 ± 20
1.4 ± 0.1	BM8	71	19	25.5	82	92	369	18	389	409 ± 28
151 ± 8	CM8	39–70	7–50	17	13–55	52–86	40–133	12	348	370 ± 20
168 ± 9	B + $CaCl_2$LM8	66	50	38	88	208	302	22	302–389	333 ± 18
197 ± 3	B + $CaCl_2$HM8	50	18.5	32	54	74	257	16	264–362	323 ± 10
216 ± 1	B + $CaSO_4$M8	74	13	29	37–108	93	245	17	330	370 ± 15
225 ± 7[b]	B + $CaCO_3$M8	117	80	51	80–120	141	184–351	24	400	393 ± 16

[a] For HPLC–UV–MS assay, the data are the averages of two values obtained from one injection of two infusions with a deviation <10%; when two values are indicated, the deviation is >10%. For ^{1}H NMR assay of CAF, the data are the means ± s.d. of three integrals for each infusion prepared in triplicate.
[b] Total calcium content after acid digestion (Mossion et al., 2008); $[Ca^{2+}]_{dissolved}$ = 15.1 ± 0.2 mg/l.

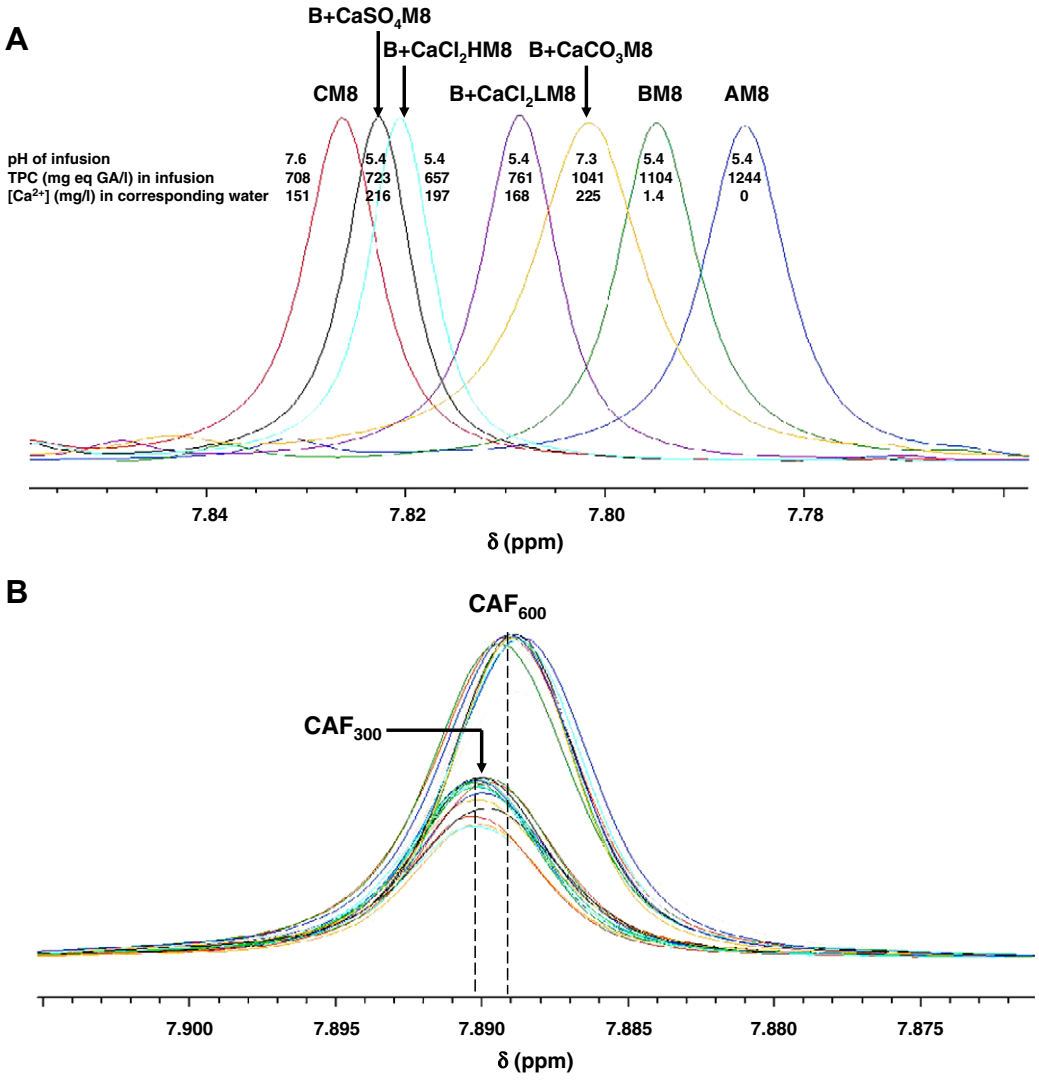

Fig. 3. Variations of H_8 δ of CAF in (A) the seven black tea infusions studied, (B) two model solutions of CAF at 300 (CAF_{300}) or 600 (CAF_{600}) mg/l prepared in waters A, B, C, B + $CaCl_2$L, B + $CaCl_2$H, at different pH between 5 and 8. Spectra were normalised to obtain the same signal intensity.

Table 4
Chemical shifts variations of CAF in model solutions and in the seven tea infusions.

		$\Delta\delta$ (Hz)			
		N_1–Me	N_3–Me	N_7–Me	H_8
Model solutions	CAF_{300}[a]	0.45	0.45	0.35	0.22
	CAF_{600}[b]	0.60	0.65	0.35	0.20
	CAF_{300}–CAF_{600}[c]	3.2 ± 1.0	2.5 ± 1.0	1.6 ± 0.7	0.5 ± 0.4
Infusions[d]	AM8	25 ± 1	22 ± 1	32 ± 1	51 ± 2
	BM8	23 ± 1	20 ± 1	30 ± 1	47 ± 2
	CM8	13 ± 1	11 ± 1	19 ± 2	30 ± 3
	B + $CaCl_2$LM8	21 ± 1	18 ± 1	26 ± 1	42 ± 2
	B + $CaCl_2$HM8	17 ± 1	15 ± 1	22 ± 1	36 ± 2
	B + $CaSO_4$M8	16 ± 1	13 ± 1	21 ± 1	34 ± 1
	B + $CaCO_3$M8	22 ± 2	19 ± 2	28 ± 3	44 ± 4

[a] 15 experiments were performed on 5 waters (A, B, C, B + $CaCl_2$L, B + $CaCl_2$H) with pH between 5 and 8.
[b] 10 experiments were performed on 5 waters (A, B, C, B + $CaCl_2$L, B + $CaCl_2$H) with pH between 5 and 8.
[c] $\Delta\delta = \delta(CAF_{300}) - \delta(CAF_{600})$.
[d] $\Delta\delta = \delta$(CAF in model solution CAF_{300}) − δ(CAF in infusion).

We thus studied CAF–polyphenol interactions in waters A and C and from H_8 δ variations. The results are reported in Table 5. Taken individually, gallate-type polyphenols (EGCg and ECg) induce the most important variations of CAF H_8 δ, 41.2 and 11.4 Hz in water A and 5.9 and 4.3 Hz in water C. The comparison with equivalent non-gallate type catechins, EGC and EC, that gives no real significant shifts confirms the binding of CAF with the galloyl group of polyphenols previously reported (Hayashi et al., 2004; Maruyama et al., 1991). It is noteworthy that no "synergy" is observed when polyphenols are mixed together as the $\Delta\delta$ measured (51.1 Hz for $\sum P_A/A$ and 12.0 Hz for $\sum P_C/C$) are close to the sum of individual changes, 55.5 Hz and 11.0 Hz, respectively (Table 5). Moreover, for water A, the $\Delta\delta$ observed for the model solution (51.1 Hz) and AM8 infusion (51 Hz) are identical, which tends to prove that only polyphenols from catechin family interact with CAF in solution. This is due to the fact that in the tea analysed, theaflavins and thearubigins are not detected as Charlton et al. (2000) have reported that CAF interacts with theaflavin. The results are quite different with water C. Indeed, the $\Delta\delta$ in the model is of 12.0 Hz while it is 30.3 Hz in the CM8 infusion, which corresponds to a difference of 18.3 Hz or 60% for CM8. Two reasons can be evoked: the degradation of polyphenols that reduces the possibility of CAF binding and/or the water composition. From data obtained for $\sum P_C$ in water A, 18 Hz *vs.* 12 Hz in water C, and $\sum P_A$ in water A, 51.1 Hz *vs.* 34.8 Hz in water C (Table 5), it can be concluded that a decrease

Table 5
Variations of the chemical shift of CAF H_8 signal as a function of polyphenols added and waters used.

	$\Delta\delta$[a] (Hz)								
	GA	C	EC	EGC	ECg	EGCg	Sum (calculated)	All polyphenols	Tea infusions
P_A in A[b]	2.1	<0.5	<0.5	0.8	11.4	41.2	55.5		
P_C in C[c]	0.8	<0.5	<0.5	<0.5	4.3	5.9	11.0		
ΣP_A in A[d]								51.1	51.0 (AM8)
ΣP_C in C[e]								12.0	30.3 (CM8)
ΣP_A in C[d]								34.8	–
ΣP_C in A[e]								18.0	–

[a] $\Delta\delta = \delta$(CAF alone in the water used) $- \delta$(CAF with polyphenol).
[b] P_A in A means that each polyphenol was individually dissolved in water A at the concentration found in the AM8 infusion.
[c] P_C in C means that each polyphenol was individually dissolved in water C at the highest concentration found in the CM8 infusion.
[d] ΣP_A in A (or C) means that the polyphenols were dissolved all together in water A (or C) at the concentrations found in AM8.
[e] ΣP_C in C (or A) means that the polyphenols were dissolved all together in water C (or A) at the highest concentrations found in CM8.

of ≈30% in polyphenol concentrations is observed when they are dissolved in water C, which means that about one third of the flavan-3-ols is degraded. However, this does not explain the difference of 60% measured between the model solution prepared with water C and the CM8 infusion. The comparison of their spectra shows that for the same CAF–polyphenol ratio, the flavan-3-ols-CAF binding is stronger in the CM8 infusion ($\Delta\delta$ = 30.3 Hz) than in the model solution ($\Delta\delta$ = 12.0 Hz). We can thus assume that both water and matrix components stabilise the binding of CAF and polyphenols in CM8.

3.6. Tea cream formation

For the seven teas analysed, precipitation (tea cream) was only observed in CM8 1 h after infusion preparation, confirming the important role of the matrix composition. Among the matrix parameters, calcium can modify surface charge of the particles and so play a role in tea cream formation (Jöbstl et al., 2005). This macroscopic effect of calcium was studied by measuring the electrophoretic mobility (ζ potential) of tea particles *vs.* pH. Three different trends were observed. AM8, BM8 and B + $CaCO_3$M8 have similar behaviour as well as B + $CaCl_2$LM8, B + $CaCl_2$HM8 and B + $CaSO_4$M8, and finally CM8. An example of each trend is shown in Fig. 4A for the three tea samples AM8, CM8 and B + $CaCl_2$HM8. Globally, all colloids are negatively charged in the range of pH studied. Moreover, the lower the pH, the higher the zeta potential. No real isoelectric point was observed, which is in agreement with previous studies (Penders, Jones, Needham, & Pelan, 1998). This behaviour is characteristic of functional groups such as carboxylic acid or phenolic functions, which can be negatively charged after deprotonation (Harbron, Ottewill, & Bee, 1989). In the present case of tea infusions, these types of sites are the dissociated hydroxyl functions of the polyphenols and those of other carboxylic acids. A similar behaviour has been reported for humic substances (Buffle, 1988). For infusions at pH 5.4 and in the range of calcium concentrations measured, zeta potential values are linearly related to calcium concentrations (R^2 = 0.9618) (Fig. 4B). This link tends to prove that calcium is able to neutralise charge particle and so to facilitate interactions by decreasing electrostatic repulsions. In the case of CM8, zeta potential is less negative than in B + $CaCl_2$HM8 as well as B + $CaCl_2$LM8, which could be related to a less important number of negative charges due to the decrease in extraction for close amount of calcium. So more charged sites are neutralised explaining that more precipitation is observed.

^{1}H NMR kinetic experiments were also performed with AM8, BM8, B + $CaCl_2$HM8 and CM8 infusions to study the role of CAF, flavan-3-ols and calcium in tea cream formation. CAF δ do not change during the recording time. This could imply that the interactions occur immediately after brewing and afterwards the particles begin to settle down. This is consistent with the hypothesis of a tea cream formation mechanism in two phases: apparition and then maturation (Jöbstl et al., 2005; Penders et al., 1998). CAF H_8 signal and polyphenol aromatic region were integrated and compared between first and last spectra. For AM8 and BM8, no significant difference was noticed in the intensity of signals of CAF and polyphenols (≈2–3% variation). For B + $CaCl_2$HM8, 48 h of kinetics were necessary to observe significant decreases of signals corresponding to CAF (10%), EGC (29%) and EGCg (23%) and a slight precipitate in the NMR tube. In the case of CM8, a large drop was measured for CAF (23%), EGC (84%) and EGCg (76%) signal intensities after only 13 h and a large precipitate was observed at the bottom of the NMR tube. These variations can be explained by

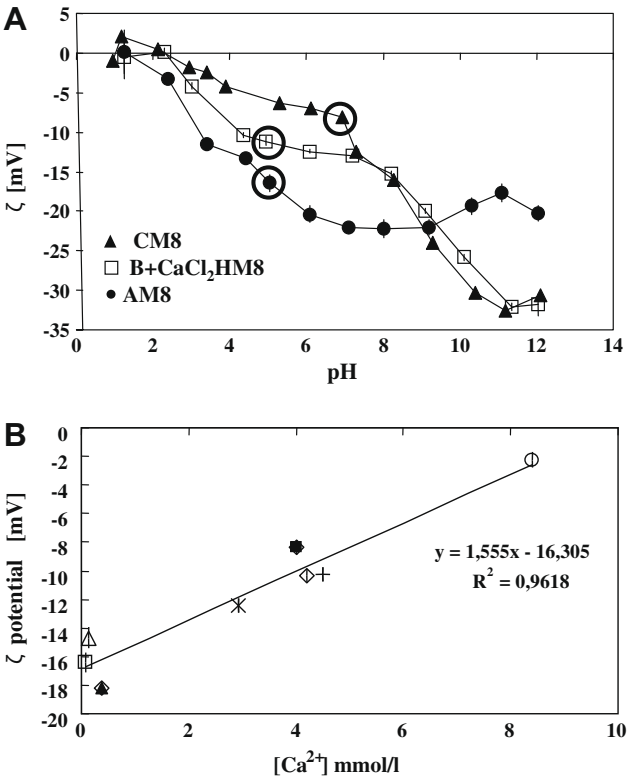

Fig. 4. (A) Variations of the zeta potential as a function of pH for three infusions, AM8, CM8 (calco-carbonated water) and B + $CaCl_2$HM8 (high calcium content water). The circled symbols represent the infusion without pH adjustment. (B) Variations of the zeta potential as a function of calcium concentrations in infusions prepared with waters A □, B Δ, B + $CaCl_2$L ×, B + $CaCl_2$H ◇, B + $CaSO_4$ +, B + $CaCl_2$400 ○ and waters C ■ and B + $CaCO_3$ ▲; linear regression was calculated only from data where infusion pH was 5.4.

polyphenol degradation for CM8 but not for B + $CaCl_2$HM8 in which flavan-3-ols are stable. Our results show that tea cream formation is a time-dependent phenomenon where more time is necessary in the case of acidic pH compared with alkaline ones. Moreover, the kinetic data seem to show that EGC and EGCg are the polyphenols that mainly interact with CAF in infusions. If EGCg–CAF interaction is known to be a major causative element in tea cream formation (Chao et al., 1999; Liang et al., 2002), this study is the first report showing that EGC (and not ECg) is implied. This shows that the galloyl group is maybe not the critical parameter for tea cream formation in the presence of calcium.

In conclusion, water calcium content decreases the extraction of organic matter during brewing and modifies interactions between CAF and polyphenols. Modelling tea infusions by solutions containing only CAF and polyphenols proved that these compounds interact in ultra pure water. For other waters, especially alkaline buffered ones controlled by calco-carbonate equilibria, the phenomenon is more complicated due to polyphenol degradation. Finally in the presence of calcium, EGC interacts more with CAF than ECg to form tea cream in real infusions.

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References

Almajano, M. P., Carbo, R., Lopez Gimenez, J. A., & Gordon, M. H. (2008). Antioxidant and antimicrobial activities of tea infusions. *Food Chemistry, 108*, 55–63.

Anderson, W., Hollins, J. G., & Bond, P. S. (1971). The composition of tea infusions examined in relation to the association between mortality and water hardness. *Journal of Hygiene Cambridge, 69*(1), 1–15.

Arce, L., Rios, A., & Valcarcel, M. (1998). Determination of anti-carcinogenic polyphenols present in green tea using capillary electrophoresis coupled to a flow injection system. *Journal of Chromatography A, 827*, 113–120.

Balayssac, S., Delsuc, M. A., Gilard, V., Prigent, Y., & Malet-Martino, M. (2009). Two-dimensional DOSY experiment with excitation sculpting water suppression for the analysis of natural and biological media. *Journal of Magnetic Resonance, 196*, 78–83.

Baxter, N. J., Williamson, M. P., Lilley, T. H., & Haslam, E. (1996). Stacking interactions between caffeine and methyl gallate. *Journal of the Chemical Society, Faraday Transactions, 92*(2), 231–234.

Buffle, J. (1988). *Complexation reactions in aquatic systems. An analytical approach*. Chichester: Ellis Horwood.

Chao, Y. C., & Chiang, B. H. (1999). Cream formation in a semifermented tea. *Journal of the Science of Food and Agriculture, 79*(13), 1767–1774.

Charlton, A. J., Davis, A. L., Jones, D. P., Lewis, J. R., Davies, A. P., Haslam, E., et al. (2000). The self-association of the black tea polyphenol theaflavin and its complexation with caffeine. *Journal of the Chemical Society, Perkin Transactions, 2*, 317–322.

Collier, P. D., Mallows, R., & Thomas, P. E. (1972). Interactions between theaflavins, flavanols and caffeine. In *Proceedings of the phytochemical society* (p. 867).

Davis, A. L., Cai, Y., & Davies, A. P. (1995). ^{1}H and ^{13}C NMR assignment of theaflavin, theaflavin monogallate and theaflavin digallate. *Magnetic Resonance in Chemistry, 33*(7), 549–552.

Erdemoglu, S. B., & Gücer, S. (2005). Selective determination of aluminium bond with tannin in tea infusion. *Analytical Sciences, 21*, 1005–1008.

Goodsall, C. W., Hodges, R. C., Jones, T. G., Mawson, J. D., & Stabler, P. J. (1999). *Tea manufacture. Application: WO Unilever Plc, UK*. Unilever NV: Hindustan Lever Limited.

Harbowy, M. E., & Balentine, D. A. (1997). Tea chemistry. *Critical Reviews in Plant Sciences, 16*(5), 415–480.

Harbron, R. S., Ottewill, R. H., & Bee, R. D. (1989). The colloid chemistry of black tea. *Special Publication – Royal Society of Chemistry, 75*(Food Colloids), 283–294.

Haslam, E. (2003). Thoughts on thearubigins. *Phytochemistry, 64*(1), 61–73.

Hayashi, N., Ujihara, T., & Kohata, K. (2004). Binding energy of tea catechin/caffeine complexes in water evaluated by titration experiments with ^{1}H-NMR. *Bioscience, Biotechnology and Biochemistry, 68*(12), 2512–2518.

Hunter, R. J. (1981). *Zeta potential in colloid science: Principles and applications*. London: Academic Press.

Jöbstl, E., Fairclough, J. P. A., Davies, A. P., & Williamson, M. P. (2005). Creaming in black tea. *Journal of Agricultural and Food Chemistry, 53*(20), 7997–8002.

Le Gall, G., Colquhoun, I. J., & Defernez, M. (2004). Metabolite profiling using ^{1}H NMR spectroscopy for quality assessment of green tea, *Camellia sinensis* (L.). *Journal of Agricultural and Food Chemistry, 52*(4), 692–700.

Liang, Y., Lu, J., & Zhang, L. (2002). Comparative study of cream in infusions of black tea and green tea [*Camellia sinensis* (L.) O. Kuntze]). *International Journal of Food Science and Technology, 37*(6), 627–634.

Martin, R., Lilley, T. H., Bailey, N. A., Falshaw, C. P., Haslam, E., Magnolato, D., et al. (1986). Polyphenol–caffeine complexation. *Journal of the Chemical Society, Chemical Communications, 10*, 5–106.

Maruyama, N., Suzuki, Y., Sakata, K., Yagi, A., & Ina, K. (1991). NMR spectroscopy and computer graphics studies on the creaming down of tea. In *Proceedings of the international symposium on tea sciences* (pp. 145-149).

Montreau, F. R. (1972). Sur le dosage des composés phénoliques totaux dans les vins par la méthode Folin–Ciocalteu. *Connaissance de la vigne et du vin, 6*, 397–404.

Morris, K. F., & Johnson, C. S. (1993). Resolution of discrete and continuous molecular size distributions by means of diffusion-ordered 2D NMR spectroscopy. *Journal of the American Chemical Society, 115*(10), 4291–4299.

Mossion, A., Potin-Gautier, M., Delerue, S., Le Hécho, I., & Behra, P. (2008). Effect of water composition on aluminium, calcium and organic carbon extraction in tea infusions. *Food Chemistry, 106*(4), 1467–1475.

Penders, M. H. G. M., Jones, D. P., Needham, D., & Pelan, E. G. (1998). Mechanistic study of equilibrium and kinetic behaviour of tea cream formation. *Food Hydrocolloids, 12*(1), 9–15.

Roberts, E. A. H. (1963). The phenolic substances of manufactured tea. X. The creaming down of tea liquors. *Journal of the Science of Food and Agriculture, 14*(10), 700–705.

Sigg, L., Behra, P., & Stumm, W. (2006). *Chimie des milieux aquatiques: chimie des eaux naturelles et des interfaces dans l'environnement* (4th ed.). Paris: Dunod.

Spiro, M., & Price, W. E. (1987). Kinetics and equilibria of tea infusion. Part 6. The effects of salts and of pH on the concentrations and partition constants of theaflavins and caffeine in Kapchorua Pekoe fannings. *Food Chemistry, 24*(1), 51–61.

Tramesel, D., Catherinot, V., & Delsuc, M. A. (2007). Modeling of NMR processing, toward efficient unattended processing of NMR experiments. *Journal of Magnetic Resonance, 188*(1), 56–67.

Trevisanato, S. I., & Kim, Y. I. (2000). Tea and health. *Nutrition Reviews, 58*(1), 1–10.

Zheng, G., Stait-Gardner, T., Anil Kumar, P. G., Torres, A. M., & Price, W. S. (2008). PGSTE-WATERGATE: An STE-based PGSE NMR sequence with excellent solvent suppression. *Journal of Magnetic Resonance, 191*(1), 159–163.

Zhu, Q. Y., Zhang, A., Tsang, D., Huang, Y., & Chen, Z.-Y. (1997). Stability of green tea catechins. *Journal of Agricultural and Food Chemistry, 45*(12), 4624–4628.