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ORIGINAL PAPER

The hydrates of chlorogenic acid in its aqueous solution and in stored food products

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Abstract

Chlorogenic acids (CQAs), a large family of naturally occurring esters of quinic acid with one, two or even three cinnamic acids moieties and/or moieties of cinnamic acids derivatives, supplied to human organisms mainly with coffee, tea, fruit and vegetables, have been among the most frequently studied polyphenols. Of these, caffeic acid esters predominate, and 5-O-caffeoylquinic acid (5-CQA) is their main and most widespread representative. Recently performed studies have shown that after removing water from the aqueous solution of 5-CQA, its dry residue contains two hydrates of this compound, each consisting of two 5-CQA molecules and two molecules of H_2O ($2\times$5-CQA*2xH_2O). The results presented in the paper not only define the characteristic features of 5-CQA but, more importantly, offer solid evidence that 5-CQA hydrates begin to form already in the aqueous solution of 5-CQA immediately after the dissolution of this compound and that the analogous process can occur in food products containing 5-CQA during their storage. Moreover, the performed experiments indicate the validity of the hypothesis that 5-CQA hydrates are formed by active collisions of 5-CQA molecules with monomeric/dimeric water molecules existing in trace amount in strongly associated aqueous systems. The presence of 5-CQA hydrates in liquid food products may have a significant impact on the assessment of some of their physicochemical properties as well as their biochemical activity.

Keywords Chlorogenic acid · Chlorogenic acid hydrates · Chlorogenic acid water complexes · Hydrates formation · Foods containing chlorogenic acid · NMR analysis

Introduction

Chlorogenic acids are a large family of naturally occurring esters of quinic acid with one, two or even three cinnamic acids moieties and/or moieties of cinnamic acid derivatives (usually with caffeic, ferulic and coumaric acid) [1, 2]. Of these, the caffeic acid esters are the predominant class. Their main and most widespread representative in nature is 5-O-caffeoylquinic acid (5-CQA)—the ester of caffeic acid with quinic acid. Due to its discovered biomedical activity combined with relatively low toxicity and side effects, potential uses of 5-CQA in pharmaceuticals and dietary products (foodstuffs, food additives) are considered [3, 4]. It is claimed that 5-CQA may help to prevent several chronic diseases, including type 2 diabetes mellitus [5], Parkinson's disease [6] and liver diseases (cirrhosis and hepatocellular carcinoma) [7, 8]. These findings cause a growing interest in the natural occurrence of CQAs and their properties [9–15].

As results from previous reports [16–20] when 5-CQA's water solution is incubated, both at room and elevated temperature, 5-CQA isomerizes and transforms into a few derivatives, two of which have molecular mass greater by 18 than the molecular mass of their parent molecule. These two compounds, at the time of their discovery, were tentatively identified as hydroxyl derivatives of 5-CQA, i.e. the products of the Michael addition of water to double bond in CQA molecules. Yet, further comprehensive NMR research of dry residues obtained after water evaporation from 5-CQA water solutions showed that the components in question forming in the residue in much greater quantities than the hydroxyl derivatives of 5-CQA, are two hydrates of 5-CQA [21], in those hydrogen bonds are created between water and OH3, OH4 and ester groups of the 5-CQA molecule.

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Recent studies [22] have not only confirmed these findings, but have also shown that each of the forming 5-CQA hydrates consists of two 5-CQA molecules and two molecules of water—2x5-CQA*2xH_2O. In these complex structures, each of the two water molecules interacting with each other via hydrogen bond forms three hydrogen bonds with one 5-CQA molecule.

Although the presence of 5-CQA hydrates in the dry residue after evaporation of water from the aqueous 5-CQA solution is in itself a new finding that extends the knowledge about the properties of chlorogenic acids, even more interesting is the fact that the amount of each 5-CQA hydrate formed in the dry residue depends on the incubation time of the 5-CQA aqueous solution [22]. The longer the incubation time, the greater is the amount of 5-CQA hydrate present in its dry residue. This indicates that 5-CQA hydrates already start forming in the aqueous solution of 5-CQA, and that their amount increases with its incubation time. The aim of the present study is not only to prove this supposition but also to demonstrate that the analogous process can occur in food products containing 5-CQA during their storage.

The results presented by Hołowiński et al. [22] suggest that hydrates of 5-CQA are formed by active collisions of properly oriented molecules of the acid with monomeric/dimeric water molecules present in a very low concentration in strongly associated 5-CQA water solution. If this mechanism is true, the partial order of the reaction of 5-CQA hydrates formation, calculated in relation to the 5-CQA concentration, should be equal to zero, i.e. independent of 5-CQA concentration. The assumption of partial order of the 5-CQA hydrate formation reaction equals zero is probable if one takes into account that the restoration rate of monomeric/dimeric water molecules in a strongly associated system is very slow and constant. Hence, the second aim of the present research work is to determine the effect of the 5-CQA concentration on the reaction kinetics of the 5-CQA hydrates formation.

Experimental

Materials and solutions used in experiments

Materials

$CuCl_2$, $Fe_2(SO_4)_3 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, HCl, CH_3COONH_4, ethanol, methanol, chloroform CH_3COONa, CH_3COOH, $Na_2HPO_4 \cdot 7H_2O$, H_3PO_4, sucrose, citric acid, NaCl, $MgCl_2$, $CaCl_2$ were supplied by the Polish Chemical Plant POCh (Gliwice, Poland). 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2′-diphenylpicrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), di-potassium peroxdisulfate, linoleic acid, neocuproine i.e. 2,9-dimethyl-1,10-phenanthroline (Nc), Tween 20, β-carotene, caffeic acid (CA), quinic acid (QA), DMSO-d_6, D_2O, caffeine, limonene, tartrazine (E102), azorubine (E122) and chlorogenic acid (5-CQA) were purchased from Sigma Aldrich (Poznan, Poland). Deionized water was purified on a Milli-Q system from Millipore (Millipore, Bedford, MA, USA).

Solutions

Two types of solutions were used in experiments:

- aqueous solutions of 5-CQA differing in 5-CQA concentration, and
- model liquid food products containing 5-CQA.

The aqueous solutions of 5-CQA were used separately for two different purposes:

- to find evidence that 5-CQA hydrates begin to form already in aqueous solutions of 5-CQA, and
- to check whether the partial order of the reaction of 5-CQA hydrate formation depends on the 5-CQA concentration.

In order to find out whether 5-CQA hydrates begin to form already in the 5-CQA solution, three aqueous solutions of the acid with a concentration of 1 mg/mL were used, both non-incubated (i.e. freshly prepared) and incubated for 1 and/or 24 h at 25 °C.

In determining the kinetics of 5-CQA hydrate formation, aqueous solutions of 5-CQA with concentrations of 0.50, 1.00, 1.50 and 2.00 mg/mL were used and incubated at 25 °C for 1, 5, 10, 15 or 24 h.

Two model liquid food products containing 5-CQA were used in these experiments:

- model isotonic drink containing 5-CQA (1 g), sucrose (50 g), NaCl (1.5 g), $MgCl_2$, (1.5 g), $CaCl_2$, (0.1 g) citric acid (0.1 g), tartrazine (E102) (0.2 g) and limonene (0.3 mL) in 1 L of water, and
- model energy drink consisting 5-CQA (1 g), sucrose (50 g), NaCl (1.5 g), $MgCl_2$, (1.5 g), $CaCl_2$, (0.1 g) citric acid (0.1 g), azorubine (E122) (0.2 g) and caffeine (0.3 g) in 1 L of water.

The above-mentioned solutions without 5-CQA, i.e. bases of model liquid food products, were also used in the experiments.

Experiments with aqueous solutions of 5-CQA and food products containing 5-CQA

To find evidence that 5-CQA hydrates begin to form already in aqueous 5-CQA solutions the following experiments were performed:

- NMR measurements of freshly prepared and incubated aqueous 5-CQA solutions and dry residues obtained from them, and
- assessment of some physicochemical properties of the 5-CQA solution incubated for different periods of time and of 5-CQA itself in those differently incubated solutions. In this experimental block, the electrical conductivity of the solutions, the susceptibility of 5-CQA to hydrolyze and the ability of 5-CQA to scavenge free radicals, to reduce metal ions and to retard and/or delay the target molecules oxidation processes were examined. In the last case, the focus was on the influence of incubation time of the aqueous solution of 5-CQA on the kinetics of radical and redox reactions occurring with 5-CQA participation. In the case of model liquid food products, only the kinetics of radical reactions were examined.

NMR measurements

Ascend 600 MHz spectrometer was employed for the NMR measurements at 298 K. Determination of CQA hydrates content in DMSO-d_6 solutions was conducted using ZG sequence for ^{1}H nuclei while the acquisition and processing of obtained spectra were carried out according to a procedure employed by Hołowiński et al. [22].To obtain pure absorption lineshapes of proton resonances in ^{1}H spectra of samples dissolved in 90% H_2O/10% D_2O, Bruker pulse program zgesgppe for excitation sculpting with perfect echo sequence for suppression of water signal was employed. 64 transients were acquired, with 1.7 s acquisition, 1 s relaxation delay and 16 ppm sweep width.

Electrical conductivity of aqueous 5-CQA solutions

Electrical conductivity of non-incubated and incubated aqueous 5-CQA solutions was estimated employing a Conductometer Model 912 (Metrohm, Poland). Between measurements, the electrodes were thoroughly rinsed with milli-Q water and dried.

Hydrolysis process of 5-CQA

The hydrolysis degree of 5-CQA contained in the non-incubated and incubated aqueous 5-CQA solutions was determined after 1 h reaction, which occurred at 25 °C in the mixture of 5-CQA solution and 0.1 M phosphate buffer, pH 3 (1:1 v/v). The amounts of CA and QA formed and the amount of 5-CQA remaining in this mixture (estimated by HPLC) were the basis for the independent calculations of the hydrolysis degree of 5-CQA.

Kinetics of radical and redox reactions of 5-CQA

Those experiments made use of classical spectrophotometric methods [23] to examine the ability of:

- freshly prepared and incubated at 25 °C for 1 and 24 h aqueous solution of 5-CQA to scavenge ABTS cation radicals and DPPH radicals, to reduce Fe(III) and Cu(II) ions and to retard β-carotene oxidation processes, and
- freshly prepared and incubated at 25 °C for 1 and 24 h model liquid food products and their bases to scavenge ABTS cation radicals and DPPH radicals.

Due to the strong antioxidant properties of 5-CQA, its aqueous solutions and model liquid food products were diluted with water just before measurement—the former 100 times and the latter 10 times.

The kinetics of ABTS cation radicals scavenging by the mentioned 5-CQA solutions and model liquid food products was estimated according to the procedure described elsewhere [24, 25]. The concentration decrease of the DPPH radicals during their reaction with those solutions was estimated by the slightly modified Brand-Williams method [26].

The degradation kinetics of β-carotene, suspended in aqueous emulsion of linoleic acid, in the presence of individual 5-CQA solutions, was performed following the procedure described by Dawidowicz and Olszowy-Tomczyk [27], whereas, FRAP and CUPRAC assays with the use of those solutions were carried out using the method described by Dawidowicz et al. [23].

In the case of each method, ABTS or DPPH or β-carotene or FRAP or CUPRAC, the quartz cuvette contained 2900 µL of radical solution or β-carotene emulsion or metal complex and 100 µL of the examined solutions containing 5-CQA. The same volume proportions were used in each case to zero the spectrophotometer, the difference being the introduction of water (for ABTS, FRAP, CUPRAC method) or methanol (for DPPH method) or aqueous emulsion of linoleic acid without β-carotene (for β-carotene method) instead of the solution containing 5-CQA.

A UV Probe-2500 Spectrophotometer (Shimadzu, Kyoto, Japan) was applied in all those procedures.

5-CQA concentration effect on the kinetics of 5-CQA hydrate formation

The effect of 5-CQA concentration on the reaction kinetics of 5-CQA hydrates formation was examined by the estimation of the 5-CQA hydrates amount in dry residues obtained after water removal from aqueous 5-CQA solutions differing in incubation time and concentration of 5-CQA. The amount of 5-CQA hydrates in the dry residue was determined analyzing its DMSO-d_6 solution by NMR.

Additional information concerning the experiments

To eliminate accidental contamination of the solutions used in the experiments by random organic and inorganic compounds, they were prepared in tight quartz tubes and the experiments reported above were performed in quartz cuvettes and vessels.

Water solution vacuum evaporation by a freeze-drying method (by lyophilization) using a CentriVap Benchtop Vacuum Concentrator (Labconco) was used to remove water from 5-CQA.

HPLC analysis

To estimate CA, QA and 5-CQA in post-hydrolytic mixtures and non-incubated and incubated 5-CQA solutions, HPLC system coupled to a quadrupole-Orbitrap mass spectrometer (Q-Exactive from Thermo Fisher Scientific, San Jose, CA) equipped with an ESI source was used. The ESI was operated in negative polarity modes under the following conditions:

- spray voltage—4.5 kV;
- sheath gas—40 arb. units;
- auxiliary gas—10 arb. units;
- sweep gas—10 arb. units and
- capillary temperature – 320 °C.

Nitrogen (> 99.98%) was employed as sheath, auxiliary and sweep gas. The scan cycle used a full-scan event at the resolution of 70,000. The column used was a 100 mm × 4.6 mm i.d., 3 µm, Gemini C18 (Phenomenex, Torrance, CA). Chromatographic separation was performed using gradient elution. Mobile phase A was 25 mM formic acid in water; mobile phase B was 25 mM formic acid in acetonitrile. The gradient program started at 5% B, increasing to 35% for 30 min, next from 35 to 100% B for 5 min, and finally isocratic elution followed (100% B) for 5 min. The total run time was 40 min at a mobile phase flow rate of 0.4 mL/min.

Statistical analysis

All results are presented as the mean value of five independent measurements ($n = 5$) ± SD. The antioxidant activities, electrical conductivity and hydrolysis degree of 5-CQA were compared using analysis of variance (ANOVA). Differences in the studied groups were considered as significant for $p \leq 0.05$ and $F_{crit} < F_{exp}$.

Results and discussion

According to Hołowiński et al. [22], the amount of 5-CQA hydrates present in the dry residue after water evaporation from an aqueous 5-CQA solution strongly depends on its incubation time: the longer it is, the greater the degree of 5-CQA conversion to its hydrates. This fact suggests that 5-CQA hydrates already start forming in an aqueous solution of the acid. As can be seen from Fig. 1A presenting the ^{1}H spectrum of a freshly prepared aqueous solution of 5-CQA, the solution contains only 5-CQA molecules. The chemical shift values characteristic for 5-CQA hydrates are not visible on this NMR spectrum. The ^{1}H spectrum of the dry residue from the freshly prepared aqueous solution of 5-CQA, dissolved in DMSO-d_6, is shown in Fig. 1B. Its analysis indicates that the residue, apart from 5-CQA, also contains 5-CQA hydrates. The chemical shift values visible in Fig. 1B are consistent with those reported by Holowinski et al. [21] for 5-CQA hydrates.

Taking into account:

- the increasing amount of 5-CQA hydrates in the dry residue obtained from longer and longer incubated aqueous 5-CQA solution reported by Hołowiński et al. [22], and
- the lack of NMR bands corresponding to 5-CQA hydrates in aqueous 5-CQA solution and the presence of hydrates in the dry residue from the solution (see Fig. 1A and B),

The experiment was repeated using an aqueous solution of 5-CQA after its 24-h incubation at 25 °C. It was presumed that the lack of visible NMR shifts corresponding to 5-CQA hydrates in a freshly prepared aqueous solution of 5-CQA could be a result of their too low concentration, as the NMR technique is characterized by a relatively low sensitivity. The ^{1}H spectra of the incubated aqueous 5-CQA solution and its dry residue obtained after water evaporation are shown in Fig. 1C and D, respectively. There are no signals in the ^{1}H spectrum indicating the presence of 5-CQA hydrates in the incubated aqueous 5-CQA solution (see Fig. 1C), whereas they are clearly visible in the spectrum for dry residue from the same solution dissolved in DMSO-d_6 (see Fig. 1D). Moreover, the concentration of 5-CQA hydrates in

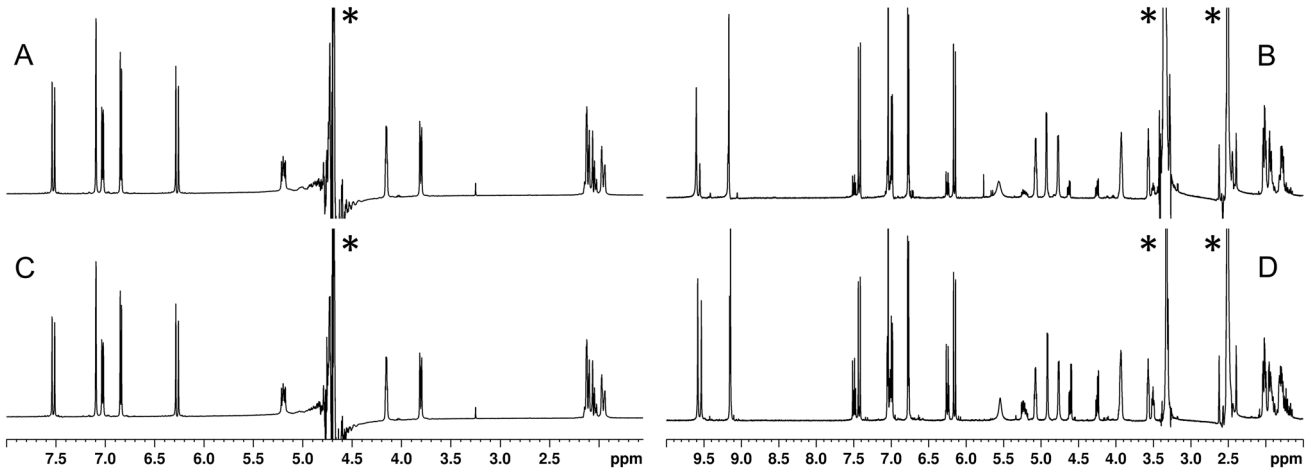

Fig. 1 ^{1}H spectra of freshly prepared (**A**) and incubated 24 h (**C**) aqueous (90% H_2O and 10% D_2O) solution of 5-CQA, and their dry residues (**B**) and (**D**), respectively, dissolved in DMSO-d_6. Solvent and residual water signals are marked by asterisks

this residue is significantly greater than that in dry residue obtained after water evaporation from the freshly prepared aqueous 5-CQA solution (compare Fig. 1B and D). The results indicate the lack of 5-CQA hydrates in the aqueous solution of the compound, unless the NMR technique is inappropriate for this purpose. However, if 5-CQA hydrates are formed already in the aqueous 5-CQA solution, and their concentration increases with the extension of incubation time, then certain physicochemical properties of the 5-CQA solution and of 5-CQA itself should change during their incubation.

A basic physicochemical quantity that seems to be helpful in reaching conclusions about formation of 5-CQA hydrates in aqueous 5-CQA solution is its electrical conductivity. The measurement of compound solution conductivity is very simple experimentally and does not destroy the molecules transporting electric charge. The influence of the incubation time of 5-CQA aqueous solution on its electrical conductivity is shown in Fig. 2A. To avoid differences due to the aging of conductometer electrodes, all conductivity values of the tested 5-CQA solutions were measured one after another with the time interval required for sample change operations. The data presented in Fig. 2 indicate a decrease in the conductivity of the aqueous 5-CQA solution with the increase of its incubation time ($F_{crit} < F_{exp}$). As stated in the introductory section, the purpose of the presented research is to provide evidence that hydrates of 5-CQA begin to form already in its solution. It should be noted here that the charge of the free 5-CQA molecule and the charge of 5-CQA in 5-CQA hydrate relating to a single 5-CQA molecule are the same. This is evidenced by the same pH of all tested 5-CQA solutions, which equals 4.09. Moreover, the incubated 5-CQA solutions are free of additional charge carriers like presumably expected CA and QA (hydrolysis products

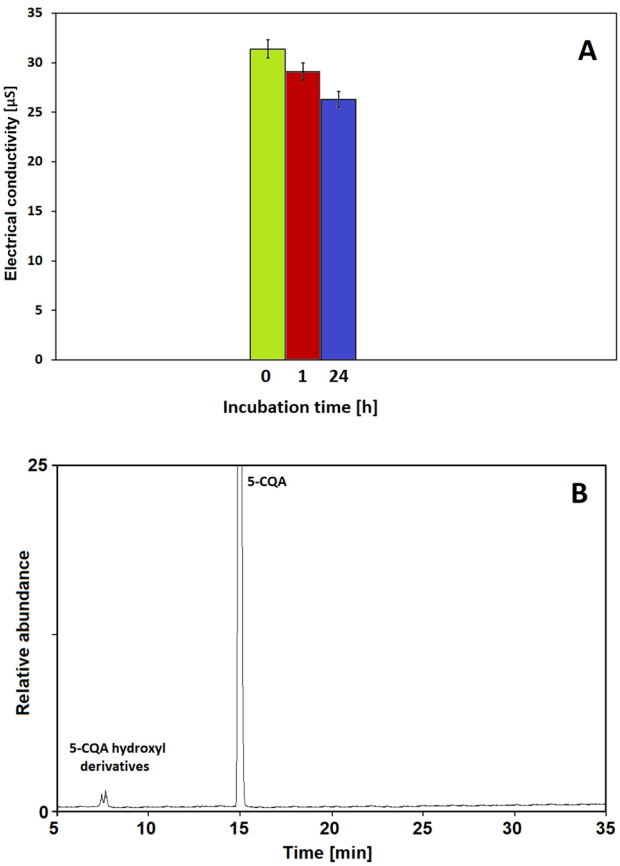

Fig. 2 The influence of incubation time of the aqueous 5-CQA solution on its electrical conductivity (**A**) and LC–MS chromatogram (in SCAN mode) of the aqueous 5-CQA solution incubated for 24 h (**B**)

of 5-CQA)—see the chromatogram in Fig. 2B corresponding to 5-CQA aqueous solution incubated for 24 h. Thus, the observed decrease in the conductivity of 5-CQA solution with the increase of its incubation time is associated with a decrease in the resultant mobility of charge carriers, which is caused by the growing number of less mobile 5-CQA hydrates in 5-CQA solution. Thus the observed decrease in the conductivity of the 5-CQA solution can be treated as indirect evidence of the formation of 5-CQA hydrates in it.

Chemically, 5-CQA, is an ester of CA with QA. The latter acids can appear in 5-CQA solution as a result of the compound hydrolysis. The hydrolysis of 5-CQA produces equimolar amounts of CA and QA. If 5-CQA hydrates are formed already in an aqueous 5-CQA solution and their concentration increases with the increase of the solution incubation time, the hydrolysis degree of 5-CQA should depend on the incubation time of its solution, assuming that the hydrolytical susceptibility of 5-CQA and its hydrates is different. An exemplary LC–MS chromatogram (in SIM mode) of the hydrolyzate obtained by partial hydrolysis of 5-CQA is presented in Fig. 3A. It shows that the hydrolyzate contains the remains of non-hydrolyzed 5-CQA and products of its hydrolysis: CA and QA. Figure 3B shows that the extension of the incubation time of 5-CQA aqueous solution increases the hydrolysis degree of the compound ($F_{crit} < F_{exp}$). Considering the larger size and lower mobility of 5-CQA hydrates than those of free 5-CQA molecules, an inverse relationship would be expected. It should be remembered, however, that the water molecules in the 5-CQA hydrate form hydrogen bonds not only with OH3 and OH4 of the 5-CQA molecule but also with its carbonyl group [21]. Consequently, the ester bond in the 5-CQA molecule that belongs to 5-CQA hydrate is more susceptible to hydrolysis than the

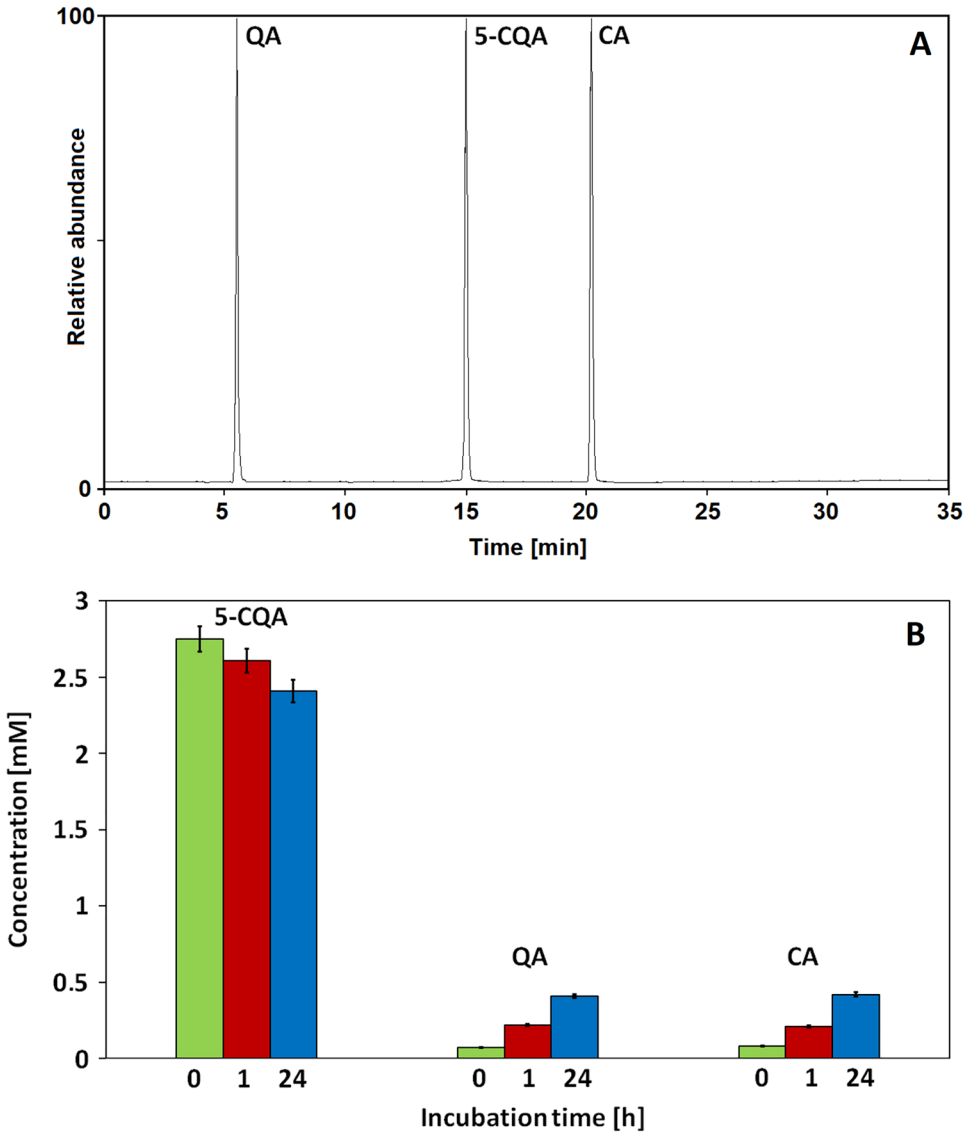

Fig. 3 Exemplary LC–MS chromatogram (in SIM mode) of the hydrolyzate of the aqueous 5-CQA solution incubated 24 h (**A**) and the concentrations of 5-CQA, CA and QA in the hydrolyzate of the aqueous 5-CQA solution, which was preliminary—non-incubated and incubated for 1 and 24 h (**B**)

ester bond in a single 5-CQA molecule not involved in the formation of such complex. Thus, the observed increase in the hydrolysis degree of 5-CQA with the increase in the incubation time of its aqueous solution results from the increase in the amount of 5-CQA hydrates forming in the aqueous solution of 5-CQA. The results shown in Fig. 3B are further indirect evidence for the formation of 5-CQA hydrates during incubation of an aqueous solution of 5-CQA.

A very important attribute of chlorogenic acids is their antioxidant properties determined, among other things, by their ability to free radical scavenging, to reduce metal ions and to retard and/or delay the target molecules oxidation processes. The antioxidant power of a compound is most often estimated by examining the kinetics of relevant reactions in which it is involved [23]. So, if 5-CQA hydrates are forming in the incubated 5-CQA solution and their amount increases with its incubation time, the kinetics of the reactions used to test the antioxidant properties of 5-CQA should change as well. Figure 4A–E. presents the kinetic curves for (1) scavenging of ABTS cation radicals and DPPH radicals (Fig. 4A and B, respectively); (2) reduction of copper (+2) ion in its complex with neocuprein and iron (+3) ion in its complex with tripyridilotriazine (Fig. 4C and D, respectively), and (3) delaying of beta-carotene oxidation (Fig. 4E) in reactions with samples of aqueous 5-CQA solutions incubated for different periods of time. The relative position of the curves indicates that the kinetics of radical reactions (see Fig. 4A and B) decreases and redox reaction (see Fig. 4C–E) increases with the extension of the incubation time of 5-CQA solution. In other words, the antioxidant power of 5-CQA solution decreases with the increase of its incubation time ($F_{crit} < F_{exp}$ calculated for values corresponding to 60 min of the reaction duration). This trend can be attributed to the transformation of 5-CQA to 5-CQA hydrates exhibiting lower antioxidant power than their precursors 5-CQA. Probably the lower antioxidant properties of 5-CQA hydrates in relation to 5-CQA itself results from poorer access of the radicals and metal complexes to their phenolic groups. The influence of hydrogen bonds present in 5-CQA hydrate on the stabilization of the phenolic radicals cannot be excluded either. It is the phenolic radicals forming in caffeic acid moiety during the reaction between phenolic groups and radicals/metal ions that are primarily responsible for the antioxidant properties of 5-CQA. Regardless of which cause for the lower antioxidant properties of 5-CQA hydrate compared to 5-CQA is more likely, the results presented in Fig. 4 are also indirect evidence of the formation of 5-CQA hydrates in an aqueous solution of 5-CQA.

In view of the presented evidence indicating the formation of 5-CQA hydrates already in an aqueous solution of the acid, the lack of explicit changes in ^{1}H spectra for aqueous CQA solutions seems surprising. This may be due to either:

- too small differences in the chemical shifts of free CQA and its complex hydrates,
- or the dynamism of processes occurring between CQA hydrates and "bulk" water or free CQA, the rate of which is too high to register by NMR the individual states involved in these transformations.

Since the 5-CQA molecules contained in an aqueous solution transform into 5-CQA hydrates, does a similar process occur in food products containing 5-CQA? A direct answer to this question seems impossible to obtain due to highly complex composition of such products and a lack of suitable research methods. Thus, in order to get at least some insight into the problem, it was decided to check the effect of storage time of a 5-CQA-containing mixture, (such mixture, which is simple in terms of composition and can be identified with a food product), on such its property that indicates the formation of 5-CQA hydrates. Model liquid food products (model energy drink and model isotonic drink) were employed as a food simulants, and an estimation of the change in the kinetics of radical reactions occurring with the use of those food simulants before and after their incubation was performed. Figure 5 presents the percent of the remaining ABTS cation radicals and DPPH radicals after their 60 min reactions with freshly prepared and incubated model isotonic drink, model energy drink and their bases. It shows that an increase in the incubation time of the used food simulants causes a decrease in their ability to scavenge radicals—the percentage of radicals remaining in the reaction/measurement system grows with the increase of incubation time. The bases of individual simulants (mixtures without 5-CQA), both freshly prepared and incubated, do not show significant ability of radicals scavenging. Therefore, the observed ability of the used food simulants to scavenge radicals and its decrease with increasing incubation time is related to the presence of 5-CQA in them and 5-CQA transformation during their incubation. As in the case of incubated aqueous 5-CQA solutions, these changes are explainable by the transformation of 5-CQA into 5-CQA hydrates.

The second aim of the present study was to respond to the suggestion made by Hołowiński et al. [22] that hydrates of 5-CQA are formed by active collisions of properly oriented molecules of the acid with monomeric/dimeric water molecules, which exist at a very low concentration in strongly associated aqueous 5-CQA solution. Figure 6A presents the influence of incubation time of differently concentrated aqueous 5-CQA solutions on the transformation degree of 5-CQA into 5-CQA hydrates, whereas Fig. 6B shows the influence of incubation time of the same solutions on the amount of unreacted 5-CQA. The analysis of Fig. 6B indicates that the reaction kinetics of the 5-CQA to 5-CQA hydrates transformation does not depends on the concentration of 5-CQA solution. It is

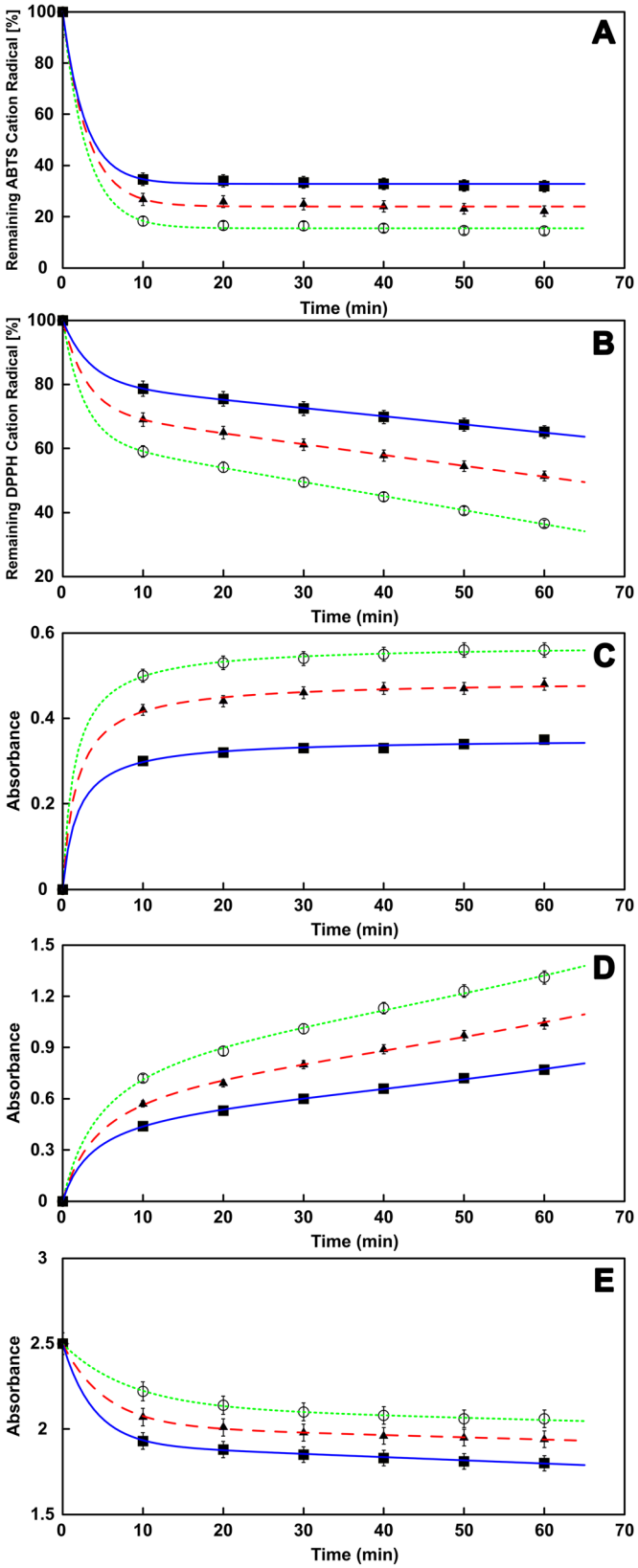

Fig. 4 Kinetic curves for reaction between ABTS cation radical (**A**) or DPPH radical (**B**) or Cu(II)-Nc complex (**C**) or Fe(III)-TPTZ complex (**D**) or peroxyl radicals (**E**) and aqueous 5-CQA solution which was non-incubated and incubated for 1 and 24 h (dotted green line with open circle, dashed red lines with triangles and solid blue line with squares, respectively)

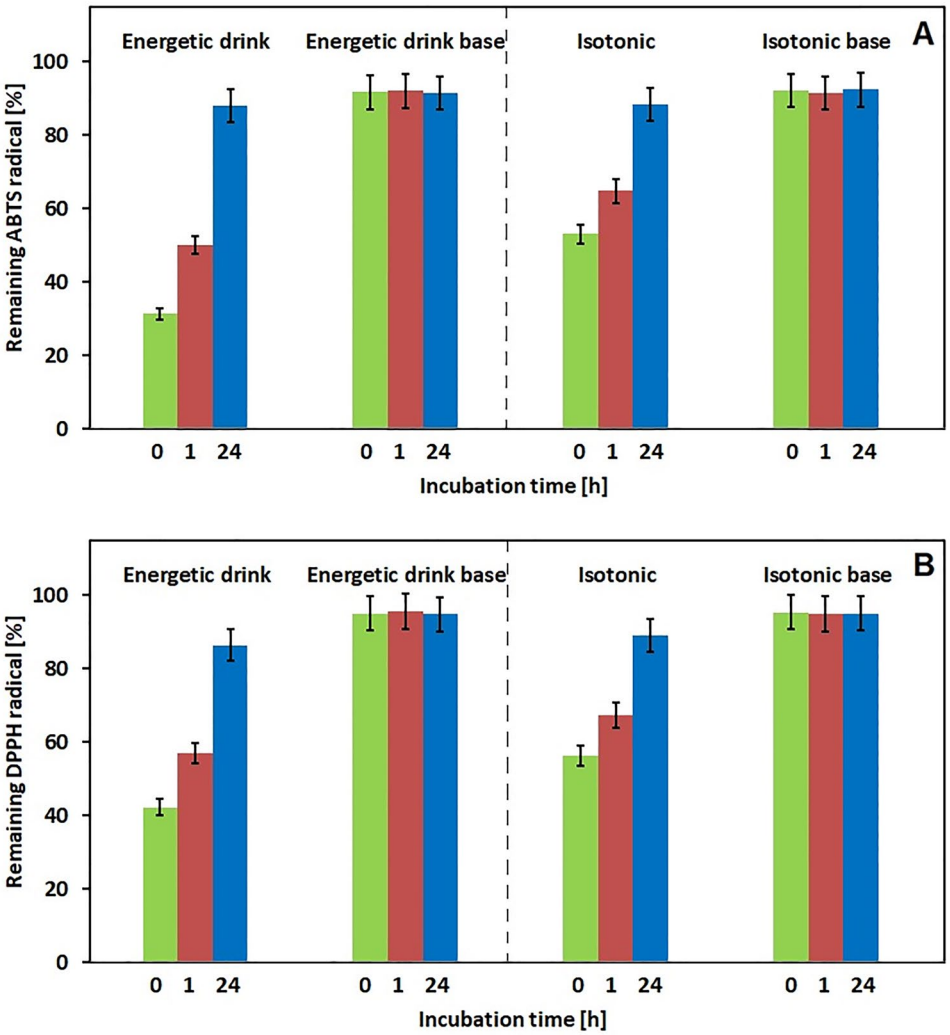

Fig. 5 The percent of remaining ABTS cation radicals (**A**) and DPPH radicals (**B**) after their 60 min reaction with freshly prepared and incubated for 1 and 24 h model isotonic fluid, model energy drink and their bases

better visible in Fig. 6C showing the influence of incubation time of aqueous 5-CQA solution on the decrease of 5-CQA concentration in four 5-CQA solutions differing in initial concentration of the acid. Its analysis shows that the loss of 5-CQA during its transformation into hydrate is constant in a given time interval and independent of the initial concentration of this acid. Despite the decreasing ratio of the moles of monomeric/dimeric water molecules to the 5-CQA moles in the aqueous 5-CQA solution, the rate of the transformation reaction is almost the same. This results from a much slower restoration of monomeric/dimeric water molecules in a highly associated aqueous solution of 5-CQA than the formation rate of 5-CQA hydrates. In consequence, the reaction partial order of 5-CQA hydrates formation calculated in relation to 5-CQA is pseudo-zero. This result confirms the validity of the model presented by Hołowiński et al.[22].

As follows from [21, 22], not only 5-O-caffeoylquinic acid, but also its isomers, 3-O-caffeoylquinic acid (3-CQA) and 4-O-caffeoylquinic acid (4-CQA) form hydrates. It can be assumed that the hydrates of latter acids also start to form already during the incubation of their aqueous solutions.

Conclusions

As results from previously [21, 22], the dry residue obtained after water evaporation from aqueous solution of 5-CQA contains 5-CQA hydrates, the amount of which strongly depends on the incubation time of the 5-CQA solution. The present paper shows that 5-CQA hydrates begin to form already in 5-CQA solution and that an analogous process can occur in food products containing 5-CQA during their storage. These statements result from changes in the physicochemical properties of 5-CQA itself, its aqueous solutions and food simulants containing the compound during their incubation. Although NMR studies do not confirm the presence of 5-CQA hydrates in aqueous 5-CQA aqueous solutions, it is likely due to very small differences in the chemical shifts of free CQA and its complex hydrates.

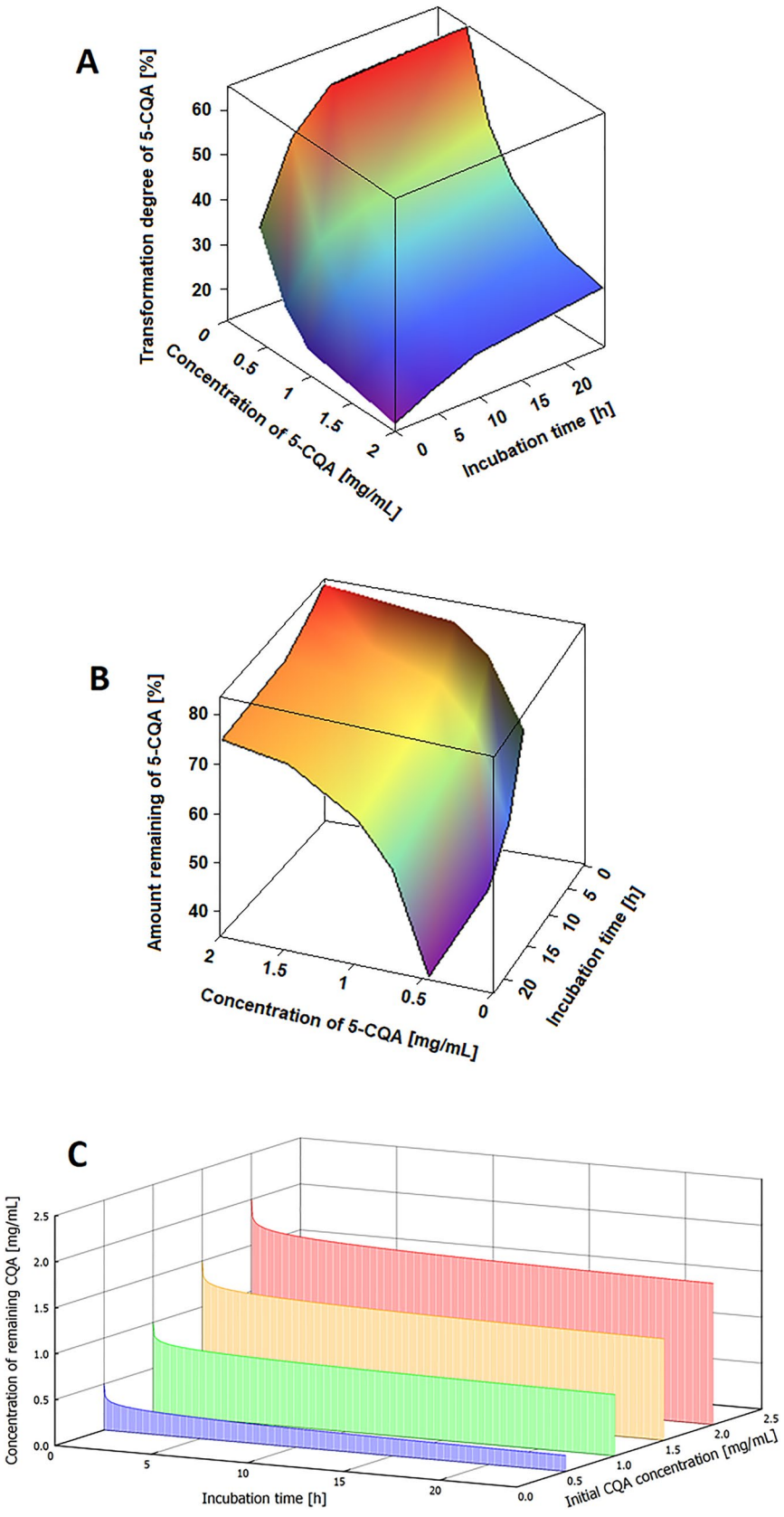

Fig. 6 The effect of incubation time of aqueous 5-CQA solutions differing in concentration on: the transformation degree of 5-CQA to 5-CQA hydrates (**A**), the percentage amount of the unreacted 5-CQA (**B**), and the amount of the unreacted 5-CQA expressed in [mg/mL] (**C**)

Another reason may be the kinetics of processes occurring between CQA hydrates and "bulk" water or free CQA, the rate of which is too high to register by NMR the individual states involved in transformation of 5-CQA to its hydrates.

The presented results not only offer solid evidence of the formation of 5-CQA hydrates in the aqueous solution of 5-CQA immediately after its dissolution but also confirm that these hydrates are formed by active collisions of 5-CQA molecules with monomeric/dimeric water molecules, as suggested previously by Hołowiński et al. [22].

Due to formation of 5-CQA hydrates in 5-CQA containing food products, some their physicochemical properties at the time of their production and after a short period of storage may be different. The difference in the physicochemical properties of 5-CQA and its hydrates may result also in differences in their biochemical properties, such as:

- different rates of their hydrolysis in the digestive tract;
- different bioavailability resulting from different ability to diffuse through biological membranes;
- different strength of their interaction with receptors.

To confirm the above the further studies are required.

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Data availability Data will be made available on request.

Declarations

Conflict of interest The authors report no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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