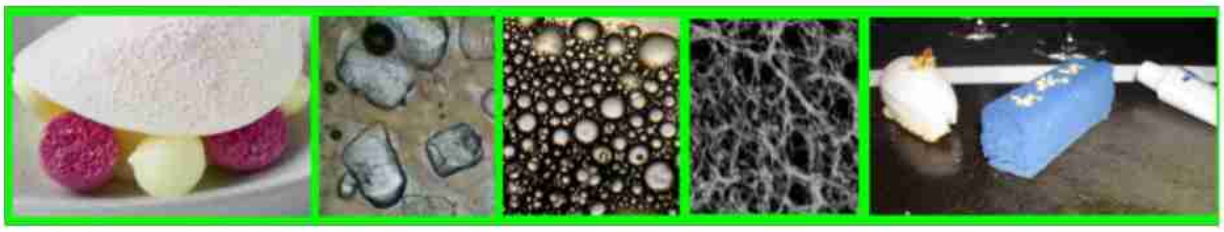

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Time-Course Evolution of Two Water-soluble Vitamins (Ascorbic acid, Nicotinic acid) and Two Amino-Acids (L-Cysteine and L-Methionine) Following Thermal Processing in Water at 100 °C (part I)

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Abstract:
Reactions of thermally processed vitamins and amino acids, in particular, ascorbic acid, nicotinic acid, L-cysteine and L-methionine were investigated in water at 100 °C. For such analysis, in situ quantitative nuclear magnetic resonance spectroscopy (*is* q ^{1}H NMR) was used which means that the samples were directly analyzed in water. This method has the advantage to be fast and non-invasive, without any extraction process. Under these experimental conditions, there were 18 % of ascorbic acid and 37 % of cysteine remaining after 96 h of reaction. Nicotinic acid and L-methionine were both found to be stable, even after 96 h of thermal processing.

Keywords:
Nutrients; time-course evolution; water; green analytical chemistry; *in situ* quantitative nuclear magnetic resonance.

Introduction

Macronutrients (proteins, amino-acids, saccharides and lipids) and micronutrients (vitamins, minerals and traces elements) are the key bioactive compounds in food#1#. During thermal treatments, their quantities in food are modified through chemical modifications and losses (compounds moving

outside of foods including the liquids from animal tissues during heat induced collagen contractions) [2,3]. In order to understand chemical modifications inside foods, it is important to study the reactivity of food compounds in conditions that mimics the internal part of food ingredients.

Vitamins and amino acids are particularly important because of their key role for good health.

Here is reported the study of two water-soluble vitamins, more precisely two organic acids, ascorbic acid and nicotinic acid. Ascorbic acid is reported to be sensitive to heat and readily oxidisable. It is involved in many biological processes in the human body such as the synthesis of collagen and of carnitine, and it is the most abundant vitamin in plant tissues (fruits and vegetables)[1,4]. Unlike ascorbic acid, nicotinic acid is described as stable to heat, light, oxidation and acid and basic conditions [5]. It acts as a co-enzyme in human metabolism and it is the most abundant vitamin in animal tissues [6]. The reactivity of a non-essential amino acid, L-cysteine, and an essential amino acid, L-methionine, were also investigated since they are reported as sensitive to oxidation and respectively a source of sulfur in human metabolism or building blocks of proteins [7,8].

In order to identify the chemical modifications of these compounds in food, the intracellular system was first modeled by heating these organic compounds alone in water at 100 °C for up to 96 hours. Water was chosen as the solvent of reaction since in food these bioactive compounds are often in the aqueous medium of the cytosol. The temperature of 100 °C was chosen because foods are mainly constituted of water, so that the inner temperature of food is generally lower than 100 °C at atmospheric pressure during home conventional heating for food production [9]. The time-course evolution of ascorbic acid in water was not much described in the food science literature. Serpen and Gökmen reported, as a control, the heating of ascorbic acid in water at 90 °C for 6 hours [10]. The loss of ascorbic acid was analyzed by high pressure liquid chromatography (HPLC) and was found to be about 72 % starting from an initial concentration of 200 mg/L. In their study on the browning phenomena of model solutions of ethanolic beverages [11], Chuang and others reported, for a standard, the heating of a solution of ascorbic acid in water (pH = 3) at 45 °C. After two days and analysis by HPLC, there is a loss of 26 % of ascorbic acid from an initial concentration of the solution of 0.5 mg/mL. Ascorbic acid could not be detected after 10 days of storage. A thorough review of the literature showed there was no study on the time-course evolution of nicotinic acid, L-cysteine or L-methionine during a thermal treatment in food or in aqueous media.

High Pressure Liquid Chromatography is the main analytical method used for the quantification of vitamins and amino acids. However, this method needs extraction steps which can be incomplete, and the development of analytical protocols can be time-consuming. In contrast, in situ quantitative proton nuclear magnetic resonance spectroscopy (*is* q ^{1}H NMR) can be helpful because it avoids any extraction process or sample preparation, since the reaction solvent is the same as the solvent used for NMR analysis. Moreover, it is a non-destructive, fast and sustainable method with a possibility of low variation coefficient. [12]

We reported here the time-course evolution of two water-soluble vitamins (L-ascorbic acid and nicotinic acid), and two amino acids (L-cysteine and L-methionine) in water at 100 °C up to five days..

Materials and Methods

Products

D_2O (99.9 %) and 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid, sodium salt (TSP-d_4; 98 %) were obtained from Aldrich (Steinheim, Germany). Ascorbic acid RP normapur was

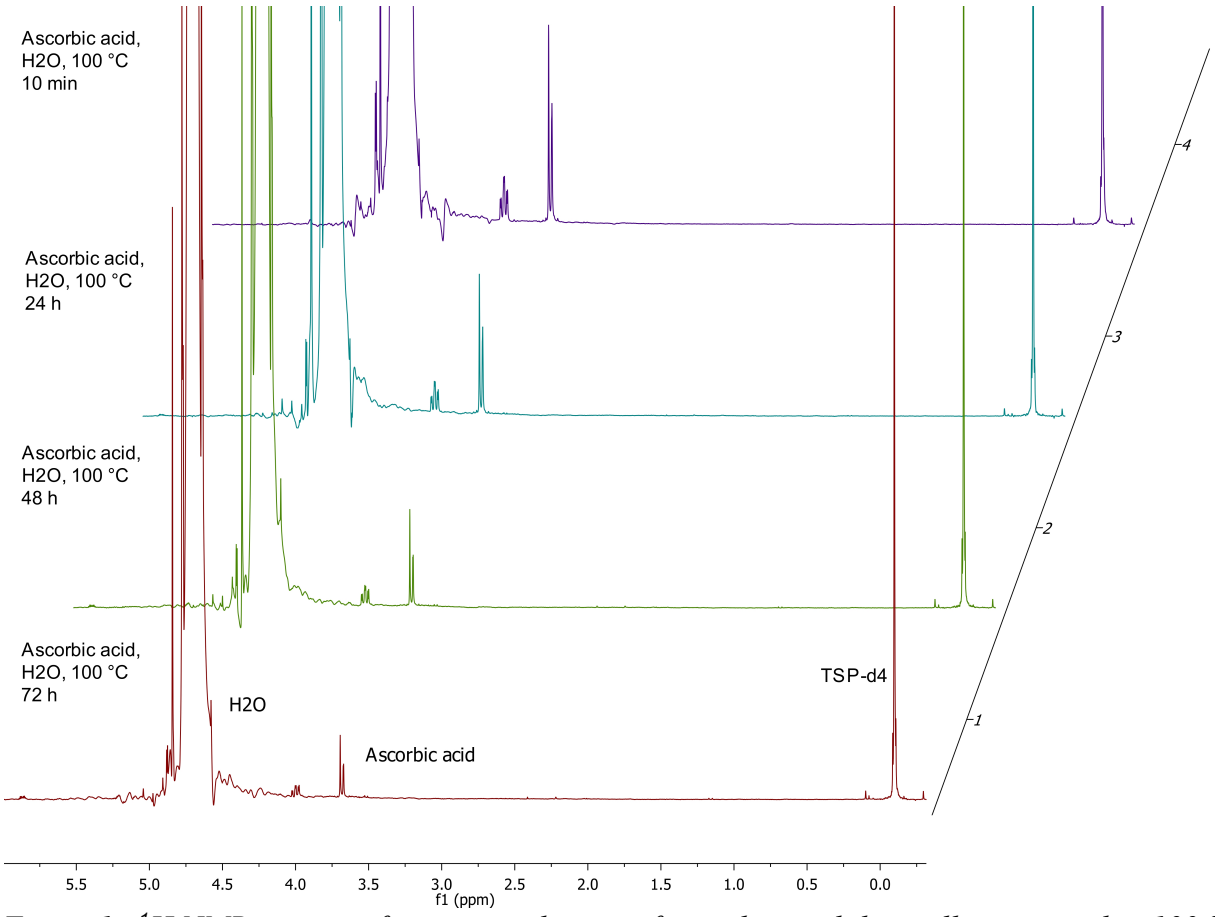

*Figure 1. ^1H NMR spectra of aqueous solutions of ascorbic acid thermally processed at 100 °C for 10 min (*top*), 24 h and 48 h (*middle*) and 72 h (*bottom*) at mass concentration C_m = 0.014 g/g. The amount of ascorbic acid decreased over five days. After 96 h, there was 18 % of ascorbic acid left in the reaction mixture, which corresponds to 2.2 mg of vitamin at 96 h over 12.5 mg at 10 min of reaction (Figure 2, (A)). We identified furfural using 1D and 2D ^1H NMR spectra since its resonances are in the aromatic zone and isolated from the other resonances.*

obtained from Prolabo, L-cysteine 97 % from Aldrich, L-methionine from Aldrich 98 %, nicotinic acid 99 % from Alfa Aesar. All samples and solutions, at each step of the analytical process, were weighed three times, using a 0.1 mg precision balance (Mettler Toledo AG 153).

NMR analysis

For NMR analysis, the *is* q NMR method was used [13]. A superconducting Ultrashield 300 MHz (7.05 T) 54 mm magnet NMR spectrometer BZH 30/300/70 E Bruker Biospin (Wissembourg, France) was used. All NMR spectra were recorded at 298 K with 32 scans of 32 K data points; they were acquired with a spectral width of 6 kHz and an acquisition time of 2.65 s. A recycle delay of 8 s per scan was set up for ascorbic acid and 18 s for L-cysteine, L-methionine and nicotinic acid per scan in order to allow complete relaxation and absolute quantification; the pulse angle was 90 °.

The aqueous solutions to be analyzed (0.7 g) were put directly in NMR tubes with a sealed capillary tube containing an aqueous solution

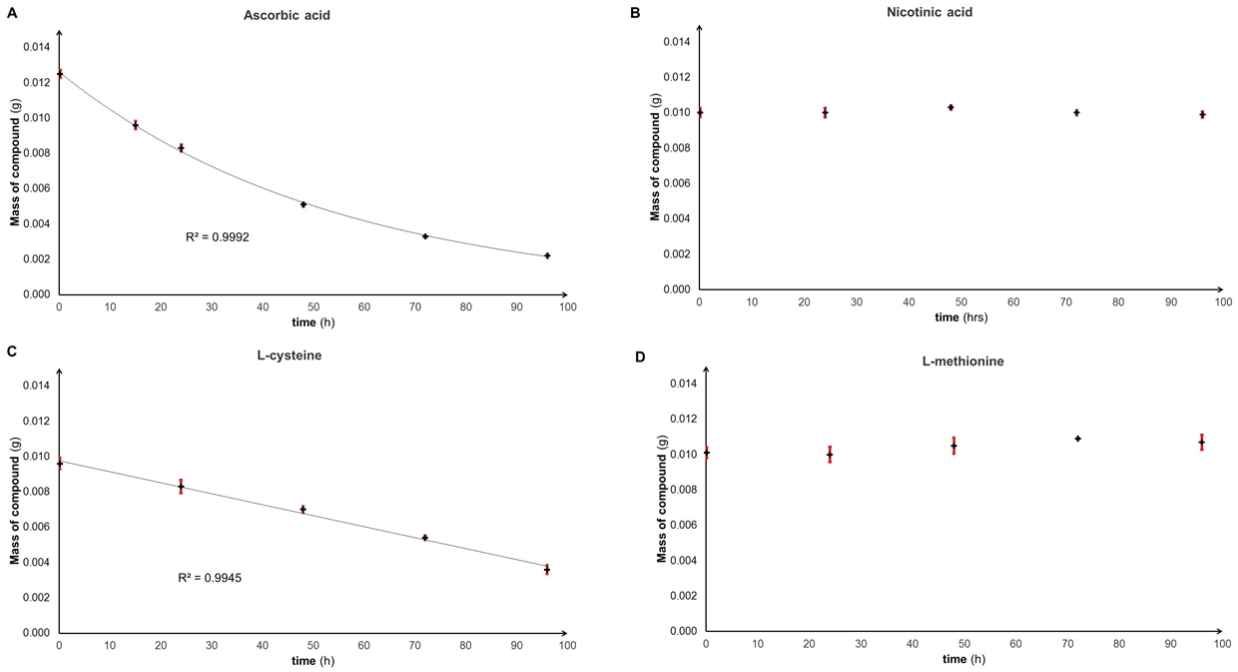

Figure 2. Time-course degradation in water at 100 °C of (A) ascorbic acid, (B) nicotinic acid, (C) L-cysteine and (D) L-methionine. Values are means for three replicates except for the point at 96 h for L-methionine which was done in duplication. The vertical bars show the standard deviation.

of TSP-d_4 in D2O (5 % of TSP). TSP-d_4 was used as internal reference and its chemical shift was set up at 0 ppm.

Spectra were acquired under an automation procedure (automatic shimming and automatic sample loading). They were Fourier-transformed with 0.3 Hz line broadening, phase and baseline were corrected using XWIN NMR 3.5 software (Bruker Biospin, Rheinstellen, Germany). The resonances in all spectra were integrated using NMR Notebook 2.7 (build 0.11, Illkirch-Graffenstaden, France) software.

For the interpretations of NMR spectra, chemical shifts (δ) are expressed in parts per million. The analysis of each sample was performed using TSP-d_4 as internal lock. For each compound, one resonance was integrated for the quantification study. We chose well separated resonance isolated from the resonance of water. Ascorbic acid was characterized using the multiplet at 3.77-3.75 ppm. L-cysteine was characterized using the multiplet at 3.17-3.01 ppm. L-methionine was characterized using the triplet at 2.65 ppm. Nicotinic acid was characterized using the multiplet at 8.11-8.06 ppm.

For quantification, the area of the TSP-d_4 resonance was used as a reference of area fixed to 1 (arbitrary units, a.u.). For each compound of interest, calibration curves were established from a mean of three calibration curves. Each calibration curve was obtained from four solutions containing a known mass of the compound of interest. Degradation curves of the processed compounds were performed using the equation of the calibration curve. All calculations were carried out using the software Maple 18 (Maplesoft, Waterloo Maple Inc, Ontario, Canada). Regression coefficient R^2 and graphs were calculated using the software Microsoft Excel 2013.

Thermal treatment of compounds

MilliQ water (14 g) was put in a 25 mL two

necked-round-bottomed flask fitted with a reflux condenser. The water was refluxed using a heater block Drysyn and 200 mg of compound were added to the boiled water. The two necked-round-bottomed flask was surrounded by aluminum foil in order to avoid heat losses and limit photochemical reactions. The reaction was refluxed up to 96 h depending of the stability of the compounds. Samples of the reaction mixture was taken, via a syringe B-Braun injekt-F fitted with a needle B-Braun Sterican, at different times according to the reaction degradation profile (0.17 h, 15 h, 24 h, 48 h, 72 h, 96 h). The first sample was carried out at 0.17 h to ensure the homogeneity of the solution. We considered that there was no degradation of compounds at 0.17 h to get the 100 % of mass of compound in the reaction mixture given the slow degradation of compounds. Samples were cooled down to room temperature before NMR analysis.
Each reaction was carried out three times to obtain statistical values.

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Time-Course Evolution of Two Water-soluble Vitamins (Ascorbic acid, Nicotinic acid) and Two Amino-Acids (L-Cysteine and L-Methionine) Following Thermal Processing in Water at 100 °C (part II)

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kinetics

Discussion:
For ascorbic acid, the data points were first arbitrarily fitted by a negative exponential curve in order to investigate the first-order reaction. R^2 was found to be equal to 0.9992, which, taking into account uncertainties, could be coherent with a first-order reaction (Figure 2, (A)).
Data were analyzed by a one way ANOVA ($p < 0.01$). Each time group was then compared with the previous time group using the Student *t* test.
A significant difference was found between means of masses at different times ($p < 0.05$) (Table 1). This profile of degradation may be explained by a low content in oxygen in water at 100 °C [14]. Indeed, ascorbic acid does not oxidize in its unstable form, dehydroascorbic acid, and thus the of degradation in anaerobic conditions is slower than in aerobic conditions.[10,15,16] The observation of traces of furfural confirms that the conditions used are anaerobic.[15,17]
The amount of L-cysteine decreased following a linear trend. The points were first fitted by a zero order reaction. R^2 was found to be equal to 0.9945 which, taking into account uncertainties, could be coherent with a zero-order reaction (Figure 2, (C)). Data were analyzed by a one way ANOVA ($p < 0.01$). Each time group was then compared with the previous time group using the Student t test. A significant difference was found between the means of masses from 48 h ($p < 0.01$) (Table 1).

Table 1. Time-course degradation of compounds of interest. a: Significantly different from T1 (Student t *Test,* p < *0.01). b : Significantly different from T2 (Student* t *Test,* p < *0.05). c : Significantly different from T3 (Student* t *Test,* p < *0.01). d : Significantly different from T4 (Student* t *Test,* p < *0.01). e : Significantly different from T5 (Student* t *Test,* p < *0.01). NS: Not Significant by a one-way ANOVA (*p > *0.05). For the calculations of percentage, we considered that there is no degradation at 0.17 hour of reaction given the slow degradation of nutrients*

Compound	Time (h)	Mass of compound (SD) (g)	Mass percentage (%) (SD)
ascorbic acid	0.17	T1 = 0.0125 (0.0005)	100
	15	T2 = 0.0096 (0.0005) ^{a}	77 (5)
	24	T3 = 0.0083 (0.0004) ^{b}	66 (5)
	48	T4 = 0.0051 (0.0002) ^{c}	41 (4)
	72	T5 = 0.0033 (0.0001) ^{d}	26 (2)
	96	T6 = 0.0022 (0.0002) ^{e}	18 (10)
nicotinic acid	0.17	T1 = 0.0100 (0.0005) ^{NS}	100
	24	T2 = 0.0100 (0.0005) ^{NS}	100 (5)
	48	T3 = 0.0103 (0.0002) ^{NS}	103 (2)
	72	T4 = 0.0100 (0.0003) ^{NS}	100 (3)
	96	T5 = 0.0099 (0.0003) ^{NS}	99 (4)
L-cysteine	0.17	T1 = 0.0096 (0.0006)	100
	24	T2 = 0.0083 (0.0008)	86 (9)
	48	T3 = 0.0070 (0.0004) ^{a}	73 (5)
	72	T4 = 0.0054 (0.0002) ^{c}	56 (5)
	96	T5 = 0.0036 (0.0005) ^{d}	38 (14)
L-methionine	0.17	T1 = 0.0101 (0.0006) ^{NS}	100
	24	T2 = 0.0100 (0.0009) ^{NS}	99 (9)
	48	T3 = 0.0105 (0.0009) ^{NS}	104 (9)
	72	T4 = 0.0109 (0.0002) ^{NS}	108 (2)
	96	T5 = 0.0107 (0.0008) ^{NS}	106 (7)

For nicotinic acid and L-methionine, the apparent absence of degradation was checked by a one way ANOVA of data. It was calculated that there is no significant difference between means ofmasses at different time (p > 0.05) (Table 1). These results are in accordance with previous studies since nicotinic acid is reported to be a stable vitamin to temperature, dioxygen and oxidizing conditions [5]. Nevertheless, to our best knowledge, the reactivity of pure L-methionine is not described in aqueous media.

In order to understand the stability of L-nicotinic acid and L-methionine, we take into account the fact that any chemical reaction is the result of breaking and making of chemical bonds. For such phenomena the thermal energy of the medium is important. Of course, depending on the particular reaction environment, two extreme possibilities can be considered: homolytic or heterolytic reactions. In both cases, the thermal energy $k_B T$ of the reaction mixture (k_B being the Boltzmann constant, and T the absolute temperature) can be compared with the bond enthalpy of

compounds being thermally processed. If the energy given by water molecules is greater than the energy of a chemical bond, the latter is broken.
For homolytic bonds, the energy required for cleavage at 298 K corresponds to the enthalpy of the reaction AB → A + B, which is by definition the bond dissociation enthalpy of the molecule AB. At 100 °C, the thermal energy $k_B T$ is of the order of 3.1 kJ/mol. The order of magnitude of energy of chemical bonds in our compounds of interest is of 400 kJ/mol which is 100 times bigger than the thermal energy.[18] This shows that the probability of breaking of the chemical bonds of the molecules of interest is low (this means that the reaction rate is about 1043 slower than the reaction rate for a thermal energy equal to the bond enthalpy, assuming an Arrhenius law).
For heterolytic bond cleavage, the energies are generally lower than for homolytic cases.
For ascorbic acid, the energy needed for breaking bond was not experimentally measured according to the literature, but the degradation of compounds similar to ascorbic acid, lactones, was studied.[19]
For such compounds, in which the ring-strained nature is a factor of destabilization (low bond energy), degradation occurs particularly through hydrolysis. Kaiser and Kézdy have shown that small structural variations in the molecules can have large effects on their reactivity, especially in β-lactones. [20] They used polarizable continuum model (PCM) calculations which take into account the contribution of solvation free energy to the total energy, and thus afford ΔG with appropriate statistical thermodynamics and solvation terms. Using this model, enthalpy values include the statistical thermodynamics enthalpic term plus the solvation free energy contribution.
They found that for β-isovalerolactone, which is the closest lactone to ascorbic acid, the energy barriers calculated for the most favorable mechanism (base catalyzed alkyl-oxygen cleavage) is 79.7 kJ/mol, whereas the experimental value is 87 kJ/mol.[21] This is more than 20 times the thermal energy.
Finally, a comparison of the thermal energy and the bond energy shows that all our compounds of interest have reasons to be stable in our experimental conditions. When instability is observed, medium effects have to be considered. In particular, solubility in water has to be taken into account for interpreting the possible destabilization and lowering of the activated complex formed from the compounds of interest and water molecules; it is well-known that such energy barrier determines the kinetic of chemical modifications.[22]

Conclusion

Quantification by isq ^{1}H NMR method allowed us to determine the half-life of isolated ascorbic acid (38 h) and L-cysteine (80 h) in water at 100 °C. No significant change of behavior of nicotinic acid or L-methionine was observed. Identification of degradation products of ascorbic acid and L-cysteine should also be carried out by ^{1}H NMR. These experiments show that more work has to be done in order to explore phenomena occurring in this simple system of aqueous solutions. *I*sq NMR is a suitable tool for studying the behavior of the starting material but more precise analytical methods are needed in order to determine the reaction products, and the possible reaction mechanisms.

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