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Research Article

Fate of ^{14}C-acrylamide in roasted and ground coffee during storage

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Acrylamide (AA) is formed during heating of carbohydrate rich foods in the course of the Maillard reaction. AA has been classified as probably carcinogenic to humans. Storage experiments with roasted coffee have shown that AA levels decrease depending on storage time and temperature. In the present study the fate of AA lost during storage of roasted and ground (R&G) coffee was studied, using ^{14}C-labeled AA as radiotracer. Radiolabel was measured in coffee brew, filter residue, and volatiles. In the brew, total ^{14}C-label decreased during storage of R&G coffee, while activity in the filter residue built up concomitantly. [2,3-^{14}C]-AA (^{14}C-AA) was the only ^{14}C-related water extractable low molecular compound in the brew detected by radio-HPLC. No formation of volatile ^{14}C-AA-related compounds was detected during storage and coffee brewing. Close to 90% of the radiolabel in the filter residue (spent R&G coffee, spent grounds) remained firmly bound to the matrix, largely resisting extraction by aqueous ammonia, ethyl acetate, chloroform, hexane, and sequential polyenzymatic digest. Furanthiols, which are abundant as aroma components in roasted coffee, have not been found to be involved in the formation of covalent AA adducts and thus do not contribute substantially to the decrease of AA during storage.

Keywords: Acrylamide / Coffee / Furfurylthiols / 3-Furfurylthiopropaneamide / 3-(5-Methylfurfurylthio-)propaneamide

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1 Introduction

Acrylamide (AA) is formed during heating of carbohydrate rich foods in the course of the Maillard reaction, depending on the heating conditions and the availability of precursors, of which asparagine is the most significant. Substantial AA amounts have been found, for example, in potato and bakery products, cereals, and roasted coffee [1 – 4]. Since AA has been classified as a genotoxic carcinogen and as probably carcinogenic to humans (group 2A) by the International Agency for Research on Cancer, Lyon (IARC) [5 – 7], mitigation of its formation in food is a priority [8]. In coffee, AA is formed during roasting. A positive correlation was found between asparagine content in the green coffee and AA content in the roasted product. During roasting, AA content reaches a maximum within the initial roasting period, followed by a steep decline toward the end of the roasting process [9]. In Europe, AA concentrations in the range of 80 – 1000 μg/kg, with a median value of 286 μg/kg [10 – 12] were reported in roasted coffee market samples [11]. In Germany, the 90th percentile for AA in roasted and ground (R&G) coffee was 277 μg/kg in the year 2007 [13]. In general, AA contents in Arabica varieties appear somewhat lower than those in robusta, probably due to a lower average asparagine content in arabica [9, 10].

Storage experiments revealed that AA levels in most matrices like cookies, cornflakes, crisp bread, and potato chips are stable during storage. In some foods however, such as roasted coffee and cocoa powder, AA levels decrease during storage depending on time and temperature [9, 12, 14]. Thus, in coffee, storage at room temperature (RT) was found to result in a consistent decrease of AA

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Abbreviations: AA, acrylamide; **^{14}C-AA,** [2,3-^{14}C]-AA; **FFT,** furfurylthiol (furan-2-yl-methanethiol); **LSC,** liquid scintillation counting; **MeFFT,** 5-methylfurfurylthiol ((5-methylfuran-2-yl)-methanethiol); **R&G,** roasted and ground; **RT,** room temperature

while storage at −18°C only marginally influenced the AA content [9]. The mechanisms underlying the loss of AA during storage as yet are unknown.

Due to its Michael reactivity, covalent binding of AA to nucleophilic groups of food components such as sulfhydryl, amino- or hydroxy groups in the food matrix [15, 16] is expected to be primarily responsible for the decrease of AA during storage. Representative reactions have already been characterized in biological systems. This applies for instance to the formation of adducts with valine in hemoglobin [17, 18], or with glutathione [18] in the organism. Such reactions have provided well established biomarkers for the monitoring of human exposure to AA at working places or through food consumption and/or as a consequence of tobacco smoke exposure. Potential reaction partners for AA might depend on the type of food in consideration. For roasted coffee, furfurylthiols, typically prevailing in amounts of 1 – 2 mg/kg [19], have been considered by us to be good candidates. Furfurylthiols have been demonstrated to degrade under oxidative conditions [20]. Moreover, they also are thought to undergo nucleophilic interactions with Michael type reactants that might be generated in the course of lipid oxidation or by Maillard-type browning reactions. The decrease reported for furfurylthiols in coffee during storage also provides support to the assumption that AA might be "used" up, at least to some extent, by its Michael reactivity, *e.g.*, toward thiol groups, including those of furfurylthiols [21].

In the present study, the fate of AA was studied in R&G coffee, using [2,3-^{14}C]-AA (^{14}C-AA) as a radiotracer. Coffee was spiked with ^{14}C-AA and then stored at RT or at 37°C for up to 48 wk. Total radiolabel, ^{14}C-AA content as well as the potential formation of ^{14}C-AA-derived reaction products were investigated in coffee brew, filter residue, and volatiles. For calculation of kinetics, a storage interval of 16 wk was considered as realistic.

Measurements were accomplished in intervals of a few weeks up to week 48. Total radioactivity in the brew was measured by liquid scintillation counting (LSC). The brew was further investigated for formation of ^{14}C-AA reaction products by HPLC, coupled with a radio flow and/or UV detector. Radioactivity in the filter residue (spent R&G coffee) was measured by LSC after combustion of the organic material to $^{14}CO_2$. Furthermore, mobilization of ^{14}C-radiolabel from the filter residue was attempted by extraction with aqueous ammonia and organic solvents and by polyenzymatic digest. Potential formation of volatile radioactive compounds during coffee storage and brewing was also investigated. Furfurylthiol (furan-2-yl-methanethiol) (FFT) and 5-methylfurfurylthiol (5-methylfuran-2-yl)methanethiol (MeFFT) prevail as major aroma compounds in fresh roasted coffee. They were therefore investigated for their ability to form covalent adducts with AA, thereby potentially contributing to nucleophilic scavenging of AA during coffee storage.

2 Materials and methods

2.1 Chemicals, reagents, and enzymes

[2,3-^{14}C]-AA (5 mCi/mmol, 1 mCi/mL ethanol) was purchased from American Radiolabeled Chemicals (St. Louis, USA), Unlabeled AA was from Merck (Darmstadt, Germany). Furfurylthiol was from Aldrich (Munich). Amylase (from *Aspergillus oryzae*) and amyloglucosidase (from *A. niger*) were from Fluka (Buchs, CH), β-amylase and β-glucosidase from Roth (Karslruhe), hesperidinase and protease (type XIV bacterial) were from Sigma (Munich), and macerozyme® (from *Rhizopus*) was from Serva (Heidelberg). All other chemicals were of analytical grade.

2.2 Syntheses

Elemental analyses were performed in the Department of Chemistry at the University of Kaiserslautern using the Perkin-Elmer 2400 CHN or the EA240 elemental analyzer. Results were within ±0.4%, unless indicated otherwise. TLC analyses were run on Alugram Sil G/UV_{254} silica gel plates (Merck). Spots were visualized under UV illumination. Column chromatography was performed on silica gel 60, 0.063 – 0.2 mm (Macherey & Nagel, Germany), flash chromatography on silica gel 60, 0.040 – 0.063 mm (Merck). NMR spectra were recorded on a Bruker AMX 400 or AMX 600 instrument using DMSO-d_6 as solvent and internal standard (^{1}H-NMR: δ = 2.49; ^{13}C-NMR: δ = 39.5). NMR signals are in ppm on δ scale.

2.2.1 5-Methylfurfuryl alcohol

Under argon atmosphere, a solution of 5-methylfurfural (6.0 mL, 60 mmol) in dry diethyl ether (240 mL) was added slowly to lithium aluminum hydride (2.32 g, 61.1 mmol) in dry diethyl ether (120 mL). The mixture was stirred and refluxed (3 h). Ice cold water (120 mL) was added and the pH adjusted to 1 – 2 by addition of 1 M H_2SO_4. The aqueous layer was extracted twice with diethyl ether (300 mL), the combined organic layers were dried with Na_2SO_4, and the solvent was removed *in vacuo*, resulting in a yellow liquid (5.68 g, 84.4%). *Rf* (ethyl acetate/hexane 1:1 v/v): 0.67. ^{1}H-NMR (400 MHz, DMSO-d_6) δ = 2.22 (d, 0.4 Hz, 3H), 4.29 (d, 5.7 Hz, 2H), 5.08 (t, 5.7 Hz, 1H), 5.96 (dd, 1.0/2.9 Hz, 1H), 6.11 (d, 2.7 Hz, 1H); ^{13}C-NMR (150 MHz, DMSO-d_6) δ = 13.5, 55.8, 106.4, 107.9, 150.9, 153.9.

2.2.2 5-Methylfurfuryl mercaptan

Under argon atmosphere, thiourea (609 mg, 8 mmol) was suspended in 0.8 M HCl (8 mL) and 5-methylfurfuryl alcohol (673 mg, 6 mmol) was added with stirring. Further stirring (4 h) provided a pale yellow precipitate. NaOH (2 M, 37.5 mL) and diethyl ether (20 mL) were added and stirring was continued (30 min). The aqueous layer was extracted twice with diethyl ether (50 mL each) to remove educt,

adjusted to pH 1–2 with HCl and extracted with diethyl ether (three times, 100 mL each). The combined organic layers were dried with Na_2SO_4 and the solvent was removed *in vacuo*, providing a yellow liquid (565 mg, 73.3%). *Rf* (ethyl acetate) 0.67. ^{1}H-NMR (400 MHz, DMSO-d_6) δ = 2.21 (s, 3H), 2.83 (s, 1H), 3.69 (s, 2H), 5.94 (d, 2.9 Hz, 1H), 6.08 (d, 2.9 Hz, 1H); ^{13}C-NMR (150 MHz, DMSO-d_6) δ = 13.5, 26.5, 106.7, 107.3, 150.9, 152.1.

2.2.3 3-Furfurylthiopropaneamide

Under argon atmosphere, AA (1.78 g, 25 mmol) and 150 mg of $Cu(BF_4)_2 6 6H_2O$ were stirred in 10 mL of furfuryl mercaptan (15.31 g, 134 mmol) for 12 h. Hexane (25 mL) was added and the yellow precipitate was filtered off and washed with few hexane and water. Flash chromatography (silica gel, ethyl acetate) afforded yellow crystals (4.38 g, 95%). *Rf* (ethyl acetate) 0.43. Anal. ($C_8H_{11}NO_2S$) C, H, N. ^{1}H-NMR (400 MHz, DMSO-d_6) δ = 2.30 (t, 7.2 Hz, 2H), 2.61 (t, 7.2 Hz, 2H), 3.75 (s, 2H), 6.26 (d, 2.5 Hz, 1H), 6.37 (dd, 2.0/3.1 Hz, 1H), 6.84 (s, 1H), 7.32 (s, 1H), 7.56 (dd, 0.8/1.8 Hz, 1H); ^{13}C-NMR (150 MHz, DMSO-d_6) δ = 27.2, 27.6, 35.5, 108.0, 111.0, 142.9, 152.1, 173.0.

2.2.4 3-(5-Methylfurfurylthio)propaneamide

Under argon atmosphere, 1.25 g (17.59 mmol) of AA was added to 2.485 g (19.35 mmol) of 5-methylfurfuryl mercaptan and 115 mg of $Cu(BF_4)_2 \cdot 6H_2O$. The mixture was stirred (12 h), producing a reddish brown suspension. The raw product was filtered off and washed with hexane and water. Column chromatography (silica gel, ethyl acetate) afforded dark yellow crystals (1.94 g, 55.4%). *Rf* (ethyl acetate) 0.39. Anal. ($C_9H_{13}NO_2S$) C, H, N. ^{1}H-NMR (400 MHz, DMSO-d_6) δ = 2.21 (s, 3H), 2.30 (t, 7.4 Hz, 2H), 2.61 (t, 7.4 Hz, 2H), 3.69 (s, 2H), 5.95 (dd, 2.0/2.9 Hz, 1H), 6.11 (d, 2.9 Hz, 1H), 6.83 (s, 1H), 7.31 (s, 1H); ^{13}C-NMR (150 MHz, DMSO-d_6) δ = 13.3, 26.7, 27.3, 35.0, 106.4, 108.3, 149.6, 150.9, 172.5.

2.2.5 Octanol/water distribution coefficient (log *P*)

Octanol and formate buffer (0.1 M) resp. PBS 1:1 v/v were saturated with the respective FFT-propaneamide and vigorously shaken for 10 min. After phase separation, each phase was analyzed for FFT-propaneamide content by UV–Vis Photometer (Varian Cary 1 Bio UV–Vis Spectrophotometer; 225 nm) and distribution was expressed as log *P* (decadic logarythm of concentration ratio of a compound distributed between octanol and aqueous phase).

2.3 Coffee

R&G arabica coffee "Brazil" (roasting degree: 88 light reflection units, colorimeter Dr. Lange; dry weight roast loss: 4.1%) was prepared by Tchibo Manufacturing, Hamburg and directly shipped. Thirty-six hours after roasting of the raw material, R&G coffee was stored at −20°C to minimize reactions between coffee components.

2.4 Preparation of ^{14}C-AA spiked coffee

R&G coffee (75 g for "high" and 150 g for "low" AA content) was slurried and stirred for 10 min in a volume of 150 mL resp. 260 mL methanolic solution containing ^{14}C-AA (0.23 μCi/mL for high spiking, 0.023 μCi/mL for low spiking). Thereafter, the solvent was removed by rotary evaporation. Total amounts of AA (intrinsic AA + added ^{14}C-AA) in two batches low spiked and in two batches high spiked R&G coffee were: 440/480 μg/kg (low) and 6400/6900 μg/kg (high). Homogeneity was checked by taking five aliquots (0.5 g each) at random from different parts of coffee and extracting each with 20 mL hot tap water (90–95°C). ^{14}C-AA activity in the aqueous extract (brew) was measured by LSC. The radiolabel was found equally distributed in the sample, with a variation of about 10% for individual measuring spots (data not shown).

2.5 Pilot experiments

2.5.1 Impact of methanol treatment on the initial AA content and decrease kinetics

The impact of methanol treatment on the kinetics of AA decrease was investigated. R&G coffee (100 g) was slurried with methanol (200 mL) and the slurry stirred for 10 min. The solvent was removed in a rotary evaporator and the sample was stored at 37°C (60 days, nonvacuum). AA determinations were commissioned to a commercial laboratory (Eurofins, Neuländer Kamp 1, 21079 Hamburg, Germany). The measurements showed that methanol treatment resulted in an initial loss of about 10% of AA but did not influence the kinetics of AA loss for the selected time period (60 days) as compared to untreated control R&G coffee (data not shown).

2.5.2 Establishing storage conditions (vacuum/nonvacuum)

In a further experiment, kinetics of AA decrease in coffee during 60 days (37°C) storage under vacuum was measured in comparison to nonvacuum storage (Tchibo Manufacturing). AA determinations were again accomplished by Eurofins. No significant difference in the decrease rates was observed between vacuum and nonvacuum conditions. Therefore, nonvacuum conditions were used for the storage experiments.

2.6 Volatility tests

2.6.1 During storage

Screening for radiolabeled components potentially volatilized during storage of ^{14}C-AA spiked R&G coffee

(25 000 cpm/5 g) was performed using an all glass closed system equipped with an activated carbon filter vent. After extraction of the charcoal filter with dichloromethane and methanol (9:1 v/v), radioactivity was measured in the extracts by LSC. In addition, wiping tests were carried out at specific points in the flask. No ^{14}C-radiolabel was found to volatilize after 4 wk storage at 37°C.

2.6.2 During extraction

R&G coffee (0.5 g), contained in an appropriate coffee filter, was inserted into a glass filter holder on top of a two necked flask equipped with an activated carbon filter vent. The flask was inserted into a water bath held at 90 – 95°C (Fig. 1). The coffee (^{14}C- activity 400 000 cpm/0.5 g) was extracted with 20 mL water (90 – 95°C). At appropriate solvent/vapor exposed positions (n = 7) wiping tests were carried out.

2.7 Coffee brew by fractionating extraction

For coffee brewing 0.5 g of ^{14}C-AA spiked R&G coffee for high and 1.0 g for low AA R&G coffee were put into a standard coffee filter paper (3 cm diameter) and extracted with 15 × 1.5 mL aliquots of hot tap water (90 – 95°C). Individual fractions 1, 2, and 3 were collected, whereas fractions 4 – 9 and 10 – 15 were pooled. Combination of fraction 1 – 9 would correspond to the preparation of a coffee drink according to a procedure recommended by "Deutscher Kaffeeverband" (Deutscher Kaffeverband http://www.deutscherkaffeeverband.de).

2.8 Analysis of total ^{14}C-activity by liquid scintillation counting

The sample (0.5 mL) was mixed with 8.5 mL scintillation cocktail (Rotiszint Eco Plus, Roth). Radioactivity was measured in a liquid scintillation counter (Beckman LS 1701) and values were taken from quenching curves prepared with coffee brew containing known amounts of ^{14}C-AA. LSC measurements were carried out 1 wk after sample preparation and addition of scintillation cocktail, to allow for decay of background fluorescence.

2.9 Combustion

The spent grounds (filter residue, spent R&G coffee) and filter paper were combusted in an oxidizer (Biological Oxidizer OX500; Harvey Instruments). The formed $^{14}CO_2$ was trapped in scintillation cocktail (10 mL Oxysolve C-400, Zinsser Analytik) and measured by LSC. Filter paper was combusted separately from filter residue. Three aliquots of the filter residue (about 100 mg each), taken from different spots of the filter cake, were subjected to combustion in cooperation with the BASF (Agricultural Center, Limburgerhof, Germany).

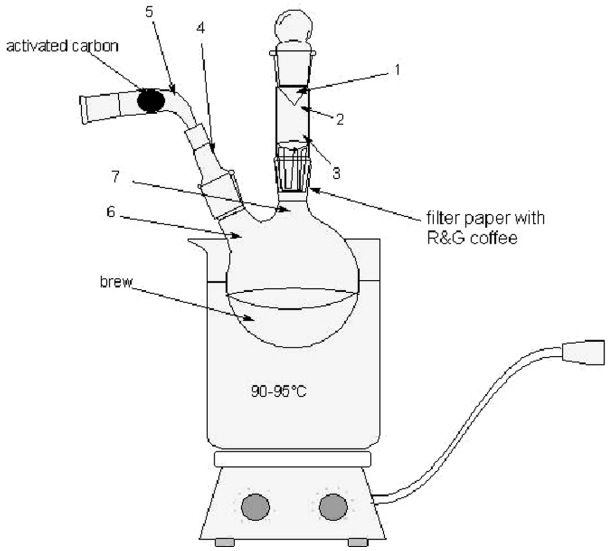

Figure 1. All glas system to investigate formation of volatiles during brewing; 1 – 7: wiping test positions.

2.10 Radio-HPLC

HPLC-system: Hitachi L-6200A Intelligent Pump (Merck). Column: Purospher STAR RP-18, end-capped, particle size 5 µm, length 250 mm, diameter 4 mm (Merck). Radio-flow detector: Radiomatic Flo-one® Beta Radio-Chromatography Detector Series A-500 (Packard). Mobile phase: gradient water/methanol (40 – 100%; 0 – 20 min) as standard system, gradient acetic acid (2.5% v/v)/ACN (3 – 90%; 0 – 50 min) for control experiments with a flow rate of 0.6 mL/min. Scintillation cocktail was Ultima-Flo M from Perkin-Elmer (Rodgau). Calibration was performed by ^{14}C-AA standard measurements with a specific activity of 5 mCi/mmol. After centrifugation, aliquots of the brew (200 µL) were injected. ^{14}C-AA was identified *via* retention time. With freshly ^{14}C-AA spiked coffee brew, total values of ^{14}C-AA activity by radio-HPLC consistently were 30% lower as compared to the corresponding total LSC values. This was taken care of by applying a correction factor to the total ^{14}C-radiodetector values.

2.11 Fractionation of the filter residue: Aqueous ammonia extraction, followed by polyenzymatic treatment

Three aliquots (50 mg) of spent grounds (filter residue, spent R&G coffee) obtained from two brewings, taken from different spots of the filter cake were extracted with 3 × 1 mL ammonia 1% v/v each and centrifuged. The pellet was neutralized with acetic acid (100%) for further incubation with enzymes or enzyme combinations (hesperidinase, β-glucosidase, macerozyme, amyloglucosidase, α/β-amylase) in appropriate buffer systems. The supernatant was subjected to acetone precipitation (24 h, 4°C acetone 1:1

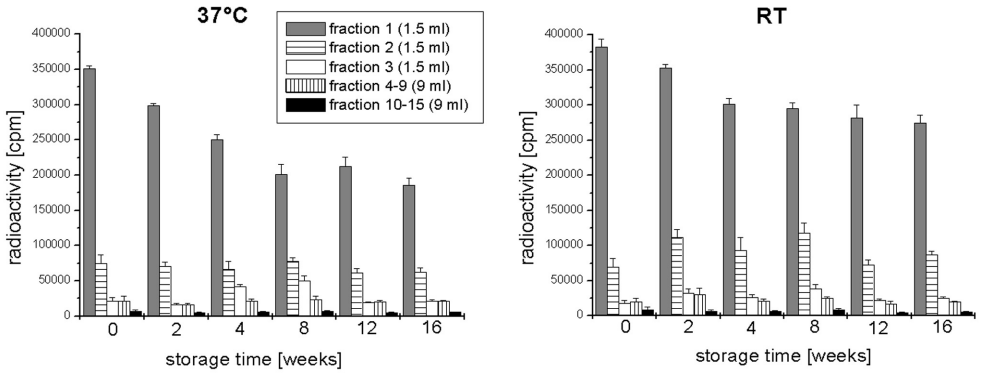

Figure 2. ^{14}C-elution profiles during coffee brewing (0.5 g R&G coffee high) by LSC up to 16 wk storage; error bars indicate SD from five independent brewings.

v/v). After centrifugation, the pellet was incubated with 2 mg protease in tris buffer (pH 7, 1.5 mL, 48 h, 37°C), the solution was subjected to LSC. The neutralized spent R&G pellet from the aqueous ammonia extraction was sequentially treated with 2 mg hesperidinase/β-glucosidase (1.5 mL acetate buffer pH 5, 48 h, 37°C), 7.5 mg macerocyme (1.5 mL acetate buffer, pH 5, 72 h, 37°C) and amyloglucosidase/α,β-amylase (7.5/2.5/2 mg, 1.5 mL phosphate buffer pH 6, 48 h, 37°C). After each enzymatic incubation, radioactivity was measured in the respective supernatant.

For extraction with organic solvents, the filter residue (spent R&G coffee, 0.1 g each) was extracted with ethyl acetate, chloroform, and hexane (1.5 mL each). Extracts were analyzed by radio HPLC for radiolabeled components.

2.12 Incubation of furanthiols with AA

Equimolar concentrations of FFT, respectively MeFFT, were incubated with AA (100 μM each) at RT in ammonium formate buffer (pH 5.5, 0.1 M) for up to 4 days under argon atmosphere. Adduct formation was determined by a Perkin-Elmer Series 200 high pressure gradient system (100×4 mm^2 RP 18 Li Chrospher, particle size 5 μM), coupled with a UV detector (UV – Vis, Perkin-Elmer 785A, 225 nm) and a PE Sciex API 2000 MS/MS System; electrospray-ionization mode (Ion source 2 kV, temperature 200°C, curtain gas 30 psi, entrance potential 10 V, declustering potential: 20 V for FFT, 25 V for MeFFT, focussing potential 400 V, gas source 1: 40 psi, gas source 2: 40 psi, solvent: ACN/formate buffer (0.1 M), pH 5.5, 60/40 – >40/60, 0 – 20 min, flow: 0.5 mL/min.

2.13 Investigation of FFT-AA in brew and filter residue

Using FFT-AA as an external reference to be recorded by HPLC/UV (225 nm) in line with radiodetection, the brew and extracts from the filter residue were examined on the same HPLC system for the presence of FFT-^{14}C-AA by radiodetection.

3 Results

3.1 Volatile formation during storage

Formation of volatiles was checked in a 4 wk storage pilot experiment using ^{14}C-AA spiked R&G coffee. No volatile ^{14}C-compounds evaporated during storage at 37°C (water bath).

3.2 ^{14}C-elution profile during coffee preparation

The ^{14}C-elution profile during filter coffee brewing was checked at specified storage time points, starting in week 0, 2, 4, and then every 4 wk up to week 16 (Fig. 2). The AA-related ^{14}C-label eluted rapidly. At all time points investigated, between 56 and 76% of the radiolabel were found already in the first fraction, eluting together with the main part of the colored components. Fraction two contained another 14 and 25% of the radioactivity, fraction three between 4 and 10%. With the model brewing procedure used, the ^{14}C-elution profile did not change significantly between coffee samples taken at different storage time points.

3.3 Coffee brew: Formation of ^{14}C-labeled volatiles during brewing

At all storage time points under investigation, no radioactive material was found to volatilize during preparation of coffee brew, as evidenced by trapping the steam developed during coffee brewing by an activated carbon filter and by wiping tests in the all glass model brewing system (Fig. 1, data not shown).

3.4 Coffee brew + filter residue: Binding of ^{14}C-label

The R&G coffee spiked with high or low amounts of ^{14}C-AA was stored for a total period of 48 wk (Fig. 3). Total radioactivity in the brew decreased with storage time. The

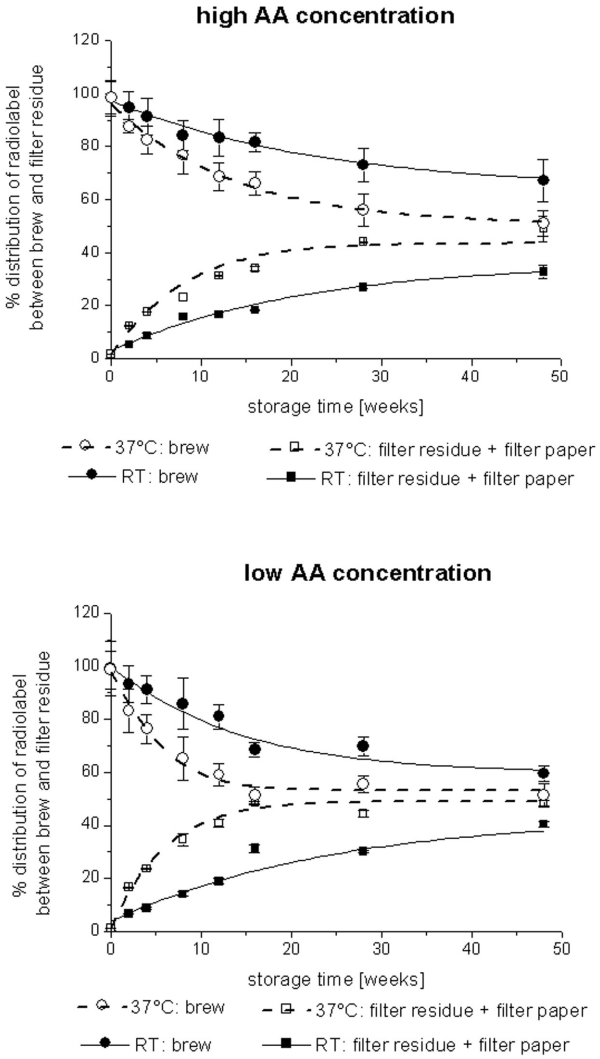

Figure 3. Variation of total ^{14}C-activity (LSC) in coffee brew and filter residue (spent coffee + filter paper) with storage at 37°C (dashed line) or RT (filled line); error bars indicate SD from five independent brewings and six filter residue combustion measurements (two independent spent coffee samples, each sample measured by three combustions; filter paper combusted separately). Curves from Boltzmann fit.

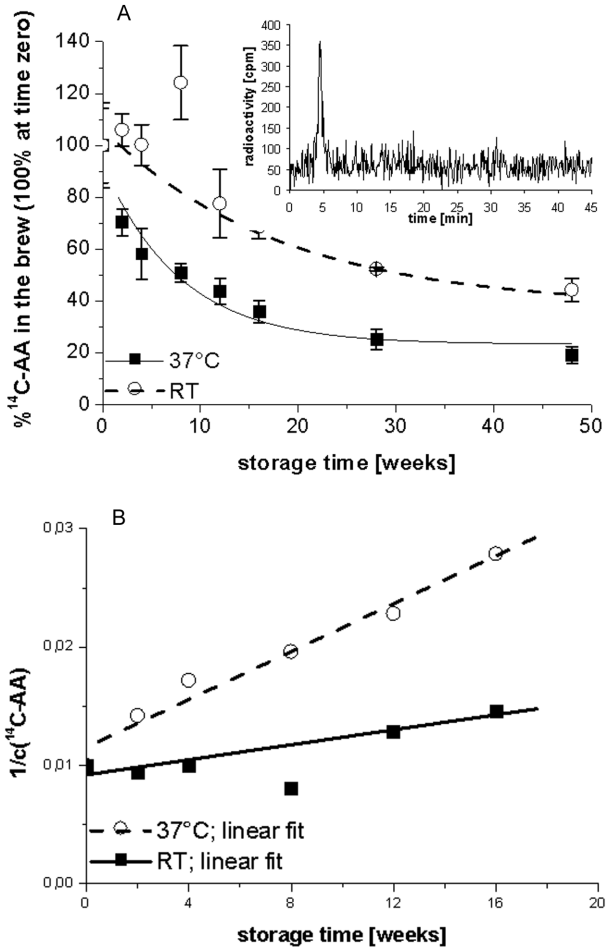

Figure 4. Analysis of coffee brew by HPLC-radiodetection.(A) Disappearance curve of ^{14}C-AA with storage time (RT: dots, dashed graph; 37°C: squares, filled graph); error bars indicate SD of three independent HPLC/radiodetection runs. Curves from Boltzmann fit. Insert: Residual ^{14}C-AA signal at week 48 (37°C). (B) Linear Fit of 1/c values of ^{14}C-AA in the brew. Rate constants taken from the slope: $k_{RT} = 3.17 \times 10^{-4} \pm 4.5 \times 10^{-5}$; $k_{37°C} = 1.01 \times 10^{-3} \pm 9 \times 10^{-5}$.

initial concentrations of ^{14}C-AA in the R&G coffee had no influence on the kinetics of ^{14}C-AA decrease. Storage at 37°C resulted in accelerated loss, compared to storage at RT. The first significant decrease (10–15%) was observed after 2 wk storage at 37°C, respectively 4 wk storage at RT. At 16 wk storage, total radioactivity had decreased down to 70% at RT for low spike (80% for high spike) and down to 50% at 37°C for low spike (65% for high spike). The corresponding values at 48 wk storage were 60% at RT and 50–55% at 37°C (high and low spike).

The ^{14}C-related label in the filter residue (extracted R&G coffee) increased time- and temperature dependently as measured by LSC after combustion to $^{14}CO_2$ (Fig. 3). Radioactivity retained in the filter paper contributed no more than 5–15% to the total label in the filter residue (data not shown). In all filter residues, ^{14}C-activity consistently and markedly increased. Maximum levels were reached after 48 wk, equalled about 50% of total label bound to the filter residue at 37°C and about 40% at RT. The corresponding values at 16 wk were 50% at 37°C for low spike (34% for high spike) and 30% at RT (18% for high spike).

Thus, the ^{14}C build up in the filter residue with storage time mirrored the ^{14}C-AA decay in the brew.

3.5 Coffee brew: Search for potential ^{14}C-AA reaction products

Under the conditions tested, only free ^{14}C-AA was detected in the brew by radio-HPLC. No indication for further water

extractable low molecular ^{14}C-labeled compounds was obtained throughout the total storage period (Fig. 4), as reflected by the invariably constant brew elution profile of samples taken at the different storage time points. However, the ^{14}C-AA-related radiolabel in the brew decayed somewhat more rapidly than the total radiolabel by LSC. After 16 wk, ^{14}C-AA content as measured by radio-HPLC had decreased down to 67% at RT (down to 36% at 37°C). At the beginning of the storage experiment, values for total radioactivity from LSC and from ^{14}C-AA (radio-HPLC, corrected) were almost identical. However, at later time points higher values for total ^{14}C-activity were found by LSC (in the brew) as compared to the values calculated from radio-HPLC signal. For example, this discrepancy reached about 10% (RT) resp. 30% (37°C) of total radioactivity in the brew at week 16 and 20%, respectively 50% at week 48. It was concluded that some material in the brew scavenges ^{14}C-AA and that the reaction products obviously escape HPLC detection. The proportion of such unidentified ^{14}C-constituents in the brew did not exceed 10% (RT) respectively 20% (37°C) of the label initially added. Derived from the ^{14}C-AA measurements, ^{14}C-AA decrease rate constants were 1.01×10^{-3}($k_{37°C}$) and 3.17×10^{-4} (k_{RT}) (Fig. 4), reflecting the binding of ^{14}C-AA to coffee components with an activation energy (E_A) of 68 ± 14 kJ/mol.

3.6 Filter residue: Search for AA binding partners

Aqueous ammonia extraction and sequential enzymatic digestion were carried out to potentially identify reaction partners responsible for ^{14}C-AA binding to the coffee matrix in the filter residue (Fig. 5). In the acetone precipitate of the aqueous ammonia extract, 2% of the label was found, potentially representing some minor proteineaceous fraction dissolved by aqueous ammonia treatment. Subsequent hesperidinase/β-glucosidase treatment of the spent ammonia-extracted residue released another 8% of the radiolabel, indicative for binding to carbohydrate structures cleavable by these hydrolases. Another 3% was found liberated into the supernatant by macerozyme treatment. Further incubation with amyloglucosidase/α/β-amylase released another

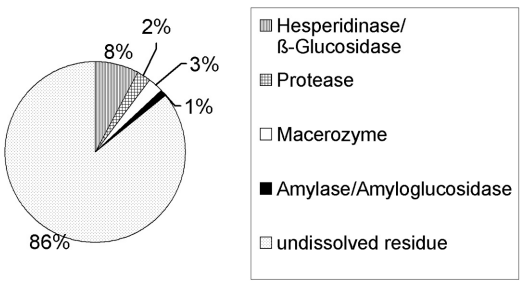

Figure 5. Distribution of ^{14}C-activity after polyenzymatic digest of the filter residue. Data from two different filter residues at 48 wk storage.

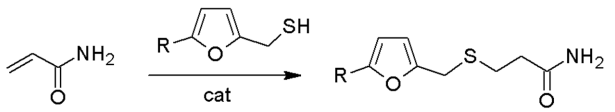

Figure 6. Synthesis of AA-furfurylthiol adducts. R = H, CH_3; cat = $Cu(II)(BF_4)_2$.

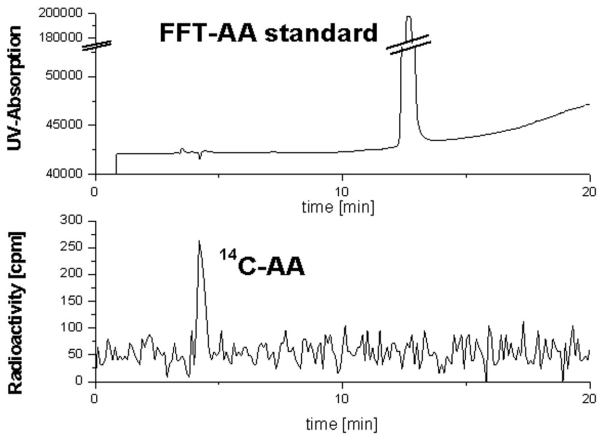

Figure 7. Search for FFT-AA adducts by HPLC-UV (225 nm) in line with HPLC-radiodetection, using FFT-AA as internal standard.

1%, indicative for binding to α/β-glucanes, such as cellulose or starch type matrix components. Overall, 86% of the label remained firmly bound to the filter residue. Moreover, extraction by organic solvents did not lead to any substantial mobilization of the radiolabel from the matrix. This clearly shows that most of the ^{14}C-AA associated radiolabel was firmly attached to insoluble matrix components, apparently as a result of covalent binding.

3.7 Synthesis of and search for AA-furanthiol-adducts

Furfurylthiol adducts of AA were synthesized *via* thia-Michael-addition in good to excellent yields, using copper(II) tetrafluoroborate as catalyst [22] (Fig. 6). Structures of the AA thiol adducts were confirmed by ^{1}H- and ^{13}C-NMR data and by elemental analyses. Exploratory incubation experiments with AA and FFT or MeFFT (100 μM each) indicated roughly 10 – 20% adduct formation at RT after 4 days (data not shown).

Octanol/PBS distribution coefficients (log P values) of 0.1 for FFT propaneamide and 0.3 for MeFFT-propaneamide indicate that these thioethers are both, hydrophilic and lipophilic, with some preference for the lipophilic octanol phase in the case of methyl FFT propaneamide. We therefore expected them to show up, at least to some extent, in the brew but also to be recovered, if formed, from the filter residue. However, neither the brew nor the organic solvent extract of the filter residue gave indications for the presence of such AA reaction products (Fig. 7).

4 Discussion

A model brewing procedure reflecting recommended household preparation of coffee brew (6 – 8 g R&G coffee brewed with 200 mL water) (Deutscher Kaffeeverband http://www.deutscherkaffeeverband.de) with standard filter paper was used to prepare brew at different storage time points.

The ^{14}C-elution profile during filter coffee brewing showed rapid elution of intact AA-related ^{14}C-label. Free AA in R&G coffee elutes essentially in the first three fractions. At all time points investigated, between 56 and 76% of the radiolabel were found in the first fraction, eluting together with the main part of the colored compounds.

With the brewing model used, the ^{14}C-elution profile did not change significantly between coffee samples taken at different storage time points.

Thus, no indication for water extractable low molecular ^{14}C-labeled reaction products was obtained throughout the total storage period, as reflected by the invariably constant brew elution profile of samples taken at different storage time points. Some evidence for further ^{14}C-AA-related radiolabel in the brew is to be taken from the fact that the ^{14}C-AA signal decayed somewhat more rapidly than the total radiolabel by LSC. Kinetics of the ^{14}C-AA decrease correlate to those reported in earlier studies [9] and to the results of a further storage experiment with the same R&G coffee spiked with unlabeled AA conducted in parallel (data not shown). In this parallel experiment 70 – 80% of free AA were recovered after 16 wk storage at RT (30% at 37°C). After 48 wk storage at RT 30 – 40% free AA remained in the R&G coffee (10 – 15% at 37°C).

Analysis of coffee brew, organic extracts of the filter residue and filter paper for FFT/MeFFT-adducts by HPLC/radiodetection gave no indication for the formation of FFT-/MeFFT-AA adducts.

Total radioactivity measurements by LSC in the brew are thought to encompass also some contribution from radiolabel due to AA reaction products not detectable by the HPLC radiodetection method utilized. In the absence of firm analytical characterization it can only be speculated that some AA-related radiolabel might be "used up" by reaction with material eluting into the brew together with the coloring material, including melanoidins or other unknown oligo/polymers such as arabinogalactans, eventually complexed to proteins/melanoidins [23] or other soluble dietary fibres [24]. Furthermore, since coffee has been reported to contain hydrogen peroxide [25] it is not unlikely that oxidation into the AA-derived epoxide glycidamide might potentially also contribute to some extent to AA loss. Thus, the chemistry and mechanisms underlying AA loss during storage deserve further elucidation.

In conclusion, the decrease of AA observed during storage of R&G coffee is to be attributed to a large extent to its covalent binding to unsoluble matrix constituents of R&G coffee. They appear to represent binding partners for AA available in excess.

Our findings show

(i) No indication for formation of volatile AA reaction products during storage or brewing.

(ii) Exclusive detection of free ^{14}C-AA in the brew.

(iii) No build up of other low molecular water soluble compound as detectable by radio-HPLC.

(iv) Built up of radiolabel in the filter residue, concomitantly with the decrease of AA in the brew.

(v) Binding of radiolabel to constituents of filter residue largely resistant to polyenzymatic digest or solvent extraction, indicative for covalent bond formation.

(vi) No indication for formation of AA furfurylthiol adducts in brew or spent filter residue.

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