1 **Enhancement of coffee brew aroma through control of the aroma staling pathway**

2 **of 2-furfurylthiol**

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Abstract:

During storage of coffee, the key aroma 2-furfurylthiol becomes less active, the mechanisms of this loss and ways to mitigate it were investigated. Aroma profiles were analyzed using GC-MS and sensory properties were evaluated by Quantitative Descriptive Analysis. Quinones, as the oxidation products of hydroxydroquinone, was found to actively bind 2-furfurylthiol, which accounted for the loss of 2-furfurylthiol. To mitigate this loss, ingredients were screened for their ability to prevent 2-furfurylthiol from loss. Cysteine had the highest 2-furfurylthiol releasing efficiency and ascorbic acid was also selected due to its 2-furfurylthiol releasing ability in Fenton reaction system. Concentrations were optimized and the addition of 0.045 g/L cysteine and 0.05 g/L ascorbic acid directly protected aroma during storage, these included 2-furfurylthiol, dimethyltrisulfide, methyl furfuryl disulfide, 4-ethylguaiacol and 4-vinylguaiacol. Ultimately, sensory testing showed a direct enhancement in nutty, sulfurous and roasted aroma attributes, an increase in flavour intensity and preference over shelf life.

Key words: 2-furfurylthiol; aroma binding; sulfur compounds; aroma release; cysteine; ascorbic acid;

1. Introduction

A strong, balanced and lasting aroma is important for the characteristic flavour profile of coffee. However, an unexpected reduction of the available aroma occurs during short time storage of the fresh coffee brew (Hofmann, Bors, & Stettmaier, 1999),

40 and negatively influences the sensory quality of coffee products (Charles-Bernard,
41 Kraehenbuehl, 2005).

42 Thiols, especially 2-furfurylthiol (2-FFT), are important for the overall aroma
43 profile of coffee. Its irreplaceability has been characterized by sensory omission studies
44 (Mayer, Czerny, & Grosch, 2000), aroma extraction dilution analysis (AEDA) and
45 odour active value determination (Hofmann & Schieberle, 2002; Peter Semmelroch &
46 Grosch, 1995). The special sulfurous-roasted odour and low threshold make 2-FFT an
47 important aroma contributor to the aroma of freshly brewed coffee (P Semmelroch,
48 Laskawy, Blank, & Grosch, 1995). However, due to its nucleophilic property, 2-FFT is
49 lost readily during storage of coffee brew (Rowe, 2009; Sun, Yang, Liu, Linforth, Zhang,
50 & Fisk, 2019).

51 The mechanism of 2-FFT loss via chemical reactions has been reported to be
52 mainly caused by ion reaction and free radical reaction (Charles-Bernard, Roberts, &
53 Kraehenbuehl, 2005; Müller & Hofmann, 2007; Weerawatanakorn, Wu, 2015). For ion
54 reaction approach, hydroxyhydroquinone (HHQ), as well as Maillard-derived
55 pyrazinium compounds, have been reported to be the potential binding precursors of 2-
56 FFT (Hofmann & Schieberle, 2002; Müller & Hofmann, 2007). Through ionic covalent
57 binding reaction, HHQ, as a mainly 2-FFT conjugate, could reversibly bind 2-FFT and,
58 subsequently, decrease the free form of 2-FFT (Müller & Hofmann, 2007; Sun, Yang,
59 Liu, Linforth, Zhang, & Fisk, 2019). As the bound 2-FFT is not sensorial-active, this
60 will negatively impact coffee aroma quality. For free radical reaction, free radicals such
61 as the hydroxyl radical cations generated by the Fenton reaction, could also lead to 2-

62 FFT degradation followed by the generation of dimers of 2-FFT (Blank, Pascual,
63 Devaud, Fay, et al., 2002). After the loss via ion reaction and free radical reaction, the
64 free 2-FFT was found to exist at trace levels in coffee (Charles-Bernard, Roberts, &
65 Kraehenbuehl, 2005).

66 Cysteine (CYS) has previously been used to prevent 2-FFT from covalent binding
67 in 2-FFT quantification method in our previous report (Sun, et al., 2018; Vichi, Jerí,
68 Cortés-Francisco, et al., 2014). Considering the nucleophilic property of mercapto
69 structure, CYS showed an ability to competitively replace the 2-FFT bound to
70 conjugates so that the 2-FFT could be released in free form again (Sun, et al., 2018).
71 For 2-FFT degradation caused by free radicals, ascorbic acid (AA) has been shown to
72 act as a hydroxyl radical scavenger (Charles-Bernard, Roberts, & Kraehenbuehl, 2005).
73 However, the 2-FFT direct binding compound and the reaction between 2-FFT and free
74 radicals are still not well documented which makes it far from solving the 2-FFT staling
75 problem.

76 Therefore, the objective of this study was to evaluate and mitigate the mechanism
77 of 2-FFT staling, ultimately to improve coffee aroma quality during storage. Initially,
78 the effect of CYS addition on HHQ concentration was investigated in a model system.
79 Following HHQ binding path, the aroma binding reactivity of quinones, as the
80 oxidation products of HHQ, was then compared with HHQ. The effect of hydroxyl
81 radicals on 2-FFT concentration was also studied in a Fenton reaction system with AA
82 addition. Consequently, a feasible solution was proposed to reduce the aroma staling in
83 stored coffee brew. Different additives (CYS, AA, methionine, sodium sulfite and

84 glutathione) were screened to evaluate their 2-FFT releasing capacity in coffee. CYS
85 and AA were selected due to their higher 2-FFT releasing capacity and their usage
86 amount was further optimized. With the addition of CYS and AA, the improvement of
87 coffee aroma quality was evaluated with instrumental analysis using GC-MS (Gas
88 Chromatography-Mass Spectrometry) and sensory tests by Quantitative Descriptive
89 Analysis (QDA). Subsequently, the correlation between volatiles concentration and
90 sensory characteristics was investigated through partial least squares regression (PLSR)
91 to reveal the key compounds of aroma preserved by CYS and AA.

92 **2. Materials and methods**

93 *2.1 Material and reagents*

94 Hydroxyhydroquinone (99%), L-cysteine, 2-furfurylthiol (98%), 1,2-
95 dichlorobenzene (99%) and ethanol were purchased from Sigma-Aldrich Co. Ltd
96 (Shanghai, China). Disodium hydrogen phosphate, sodium dihydrogen phosphate,
97 sodium sulfite, iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), iron (II) sulfate
98 monohydrate ($FeSO_4 \cdot H_2O$), hydrogen peroxide (H_2O_2, 30%), egg albumin,
99 ethylenediaminetetraacetic acid (EDTA, disodium salt), ethyl acetate, ascorbic acid,
100 methionine and glutathione (99%) were purchased from Sinopharm Chemical Reagent
101 Co. Ltd (Shanghai, China). Millipore water was made by Ultrapure Water Maker Type
102 1 (Weston, USA). Green Robusta coffee beans (sun drying, crop years 2016/2017) from
103 Vietnam was purchased from Royal Coffee Co. Ltd (Shanghai, China).

104 *2.2 Sample Preparation and preparation methods*

105 *2.2.1 Coffee roast and fresh coffee brew preparation*

106 Raw Robusta coffee beans of 90 g were roasted by a CR-100 Coffee Roaster
107 (Genesis, Korea) at 220 °C for 12.5 min. Color of the roasted beans based on L^*
108 (lightness), a^* (+ red, - green), and b^* (+ yellow, - blue) was analyzed by UltraScan Pro
109 (Hunterlab, USA). The results ($L^* = 40 \pm 0.32$, $a^* = 3.84 \pm 0.1$, $b^* = 3.84 \pm 0.12$)
110 indicated the beans had been roasted to medium roast level (Mendes, de Menezes, et
111 al., 2001).

112 The roasted beans were ground using a coffee grinder (KG 49, Delonghi, Australia)
113 and screened by a sieve (700 mm). Nine grams ground coffee was extracted using
114 deionized water (180 mL) at 92 °C for 4 min by a French press brewer (Hario, Japan).
115 The temperature of extracts at the brewing end was around 85 °C. After brewing, the
116 coffee extract was filled into amber vials of 40 mL sealed by screwed top Teflon septa
117 (Supelco, USA) and cooled to 40 °C by a water bath.

118 *2.2.2 The effect of* CYS *addition on the concentration of hydroxyhydroquinone in model*
119 *system*

120 The phosphate buffer solution of 0.1 M at pH 6.0 was established by the solution
121 containing sodium dihydrogen phosphate and disodium phosphate. 2-FFT (3.6 μg in 10
122 μL of methanol) was added into the 40 mL vials and filled by buffer solution with 0.1
123 g egg albumin, which was reacted with 0.1 mg HHQ under incubation at 40 °C for 30
124 min (Mottram, Szauman-Szumski, & Dodson, 1996). Samples were sealed by screw
125 top with Teflon cover. After incubation, CYS (0, 0.08, 0.24, 0.32, 0.48 and 0.64 g) was
126 added into the buffer solution, mixed by magnetic stirring (IKA RET control-visc, USA)
127 at 500 rpm for 5 min. The concentration of HHQ and 2-FFT were analyzed by liquid

128 chromatography mass spectrometry (LC-MS) and solid phase microextraction-gas
129 chromatography-mass spectrometry respectively (SPME-GC-MS).

130 *2.2.3 Evaluation of the stability of 2-furfurylthiol incubated with hydroxyhydroquinone*
131 *or the oxidation product of hydroxyhydroquinone before and after sodium sulfite*
132 *addition in model system*

133 To mimic the binding behavior of 2-FFT in brewed coffee, three different potential
134 2-FFT binding reactants were placed in the buffer solution respectively. The reactants
135 were HHQ (0.1 mg), the oxidation products of HHQ, and the oxidation products with
136 0.01 g sodium sulfite. The oxidation products of HHQ were prepared as follows: 0.1
137 mg HHQ was diluted into the 40 mL buffer solution, and then incubated with 0.14 mg
138 $FeSO_4 \cdot H_2O$ and 0.27 mg EDTA at 40 °C for 30 min (Cilliers & Singleton, 1989; Frostin-
139 Rio, Pujol, et al., 1984; Mueller, Hemmersbach, Van't Slot, et al., 2006).

140 2-FFT (3.6 μg in 10 μL of methanol) was then stored with the respective reactants
141 in the buffer solution of 40 mL at 40 °C water bath for 40 min. The concentration of 2-
142 FFT was analyzed by SPME-GC-MS.

143 *2.2.4 Fenton Reaction model preparation*

144 Fenton Reaction model was modified based on Blank's report (Blank, et al., 2002).
145 Phosphate buffer solution of pH 6.0 was placed in sample vials of 40 mL with H_2O_2 (20
146 μL), $FeCl_3 \cdot 6H_2O$ (1 mg), EDTA (19 mg) and 2-FFT (3.6 μg in 10 μL of methanol). AA
147 (0, 0.015, 0.03, 0.06, 0.12 g/L) was added into the 40 mL buffer samples respectively
148 and stored at 40 °C water bath for 1 h.

149 *2.2.5 The optimization of cysteine and ascorbic acid added amount in coffee brew*

150 *Cysteine usage amount optimization:* The addition of CYS was adjusted to
151 minimize the off-flavor it generated by sensory test since the over supplement of CYS
152 would generate uncoordinated smell in coffee (Kobayasi & Fujimaki, 1965). The off-
153 flavor threshold was detected using the forced-choice ascending method of limits (Intl,
154 2011; Meilgaard, Carr, & Civille, 1999). All the sensory evaluation tests were
155 conducted in the consequent morning at the sensory laboratory (ISO-Standard 8589,
156 2007) of Jiangnan University (Wuxi, China). Fifteen panelists (eight females and seven
157 males from age 20 to 35) were chosen from the postgraduate students in the School of
158 Food Science. They were trained before further experiment: Every panelist was set in
159 an individual testing booth. Panelists were first asked to evaluate the smell of CYS
160 solution (8 mg CYS in 40 mL Millipore water at 60 °C) and give descriptive words to
161 help them recognize the smell of CYS in solution system. In the training, two triangle
162 tests were used with or without addition of CYS (Series 1: 0, 0, and 4 mg; Series 2: 0,
163 0, and 8 mg) into the 40 mL coffee. Panelists were asked if they could detect the
164 difference between the samples in these two sets and they also wrote down the odour
165 description for the CYS smell in samples after the triangle tests. Then the panelists were
166 given the correct answers and were instructed to smell again to be familiar with the off-
167 flavor.

168 The determination of the threshold of off-flavor after CYS addition in coffee brew
169 was performed using the three-alternative forced-choice (3-AFC) test. In this test, each
170 panelist was presented 18 samples, corresponding to six 3-AFCs with six dilution levels
171 using 1.2, 1.6, 2.0, 2.4, 2.8 and 3.2 mg CYS mixed in 40 mL coffee with magnetic

172 stirring at 1200 rpm for 1 min. Every 3-AFC sample included two blank coffee samples
173 and one CYS added sample at randomized orders. In each 3-AFC sample set, panelists
174 were instructed there were two blank samples and one CYS added sample. During the
175 test, panelists were asked to uncover the samples, smell and told to choose the sample
176 added CYS after smell tests. The threshold for CYS was obtained according to
177 American Society for Testing and Material (ASTM, 2011): The individual threshold
178 (the best estimates threshold, BET) was the geometric mean between the highest missed
179 concentration and the next adjacent higher concentration. The group threshold level for
180 CYS was the geometric mean of all equivalent amounts.

181 *Ascorbic acid usage amount optimization:* the AA used was optimized based on
182 the 2-FFT concentration in coffee brew after incubation. The AA was presented into the
183 40 mL amber-vial samples at five levels (0.03, 0.04, 0.05, 0.06, 0.07 g/L) with the
184 optimal CYS concentration (obtained by the following sensory evaluation) and
185 incubated for up to 30 min at 40 °C (Sun, et al., 2018). The concentration of 2-FFT was
186 analyzed by SPME-GC-MS.

187 *2.2.6 Sensory evaluation of aroma profile of the coffee with cysteine and ascorbic acid*
188 *addition*

189 The sensory evaluation on the aroma profile of the coffee was investigated by
190 quantitative descriptive analysis (QDA) (Stone, Sidel, Oliver, Woolsey, & Singleton,
191 2008). The panel was composed by 10 assessors (five females and five males from age
192 20 to 35) and they have received over 20 h training containing theoretical education,
193 general sensory evaluation and descriptive sensory training of coffee. The descriptors

194 were generated from the trained panelists and modified based on coffee flavor wheel
195 (The Specialty Coffee Association of Europe, 2016) and previous reports (Hanseok,
196 Seungyeon, & Inkyeong, 2010; Narain, Paterson, & Reid, 2004). Nine descriptors
197 including (nutty, flowery, fruity, sulfurous, roasted, spicy, chocolaty, preference, aroma
198 intensity) were evaluated by 0 to 10 scores, indicating the intensity of aroma
199 characteristics from imperceptible to very intense.

200 During the sensory evaluation, fresh brewed coffee of 40 mL was placed in glass
201 cups (60 mL, 35 mm × 41 mm) with a three-digit number and incubated for 0, 15 and
202 30 min at 40 °C with CYS (0.045 g/L), or AA (0.05 g/L) and CYS (0.045 g/L), or
203 without additives. Samples were served to panelists at 40 °C. There were 3-min rest
204 intervals between samples and 5-min rest after the 4th sample to minimize the sensory
205 fatigue (Bleibaum, Stone, Tan, et al., 2002; Kreuml, Majchrzak, Ploederl, & Koenig,
206 2013).

207 *2.2.7 Identification and quantification of volatile compounds*

208 Volatiles were identified by comparing their detected mass spectra with the
209 authentic standard mass spectra or data libraries (NIST 11 and WILEY 07 databases).
210 The Kováts retention index of each detected compound was calculated by a
211 homogenous series of n-alkanes (C6-C26) standard in the same GC condition and
212 compared with references.

213 The quantification of 2-FFT in the model system was presented by external
214 standard method. The standard in buffer solution was prepared from 10 to 200 μg/L,
215 and the standard curve was obtained ($y = 4034.7x - 4213.8$, $R^2 = 0.999$).

216 The specific quantification of 2-FFT in coffee (Item 2.6 to 2.8) was performed
217 based on our previous report (Sun, et al., 2018). The 2-FFT standard curve was prepared
218 by diluting standard in a prefabricated coffee model at 10, 50, 100, 150 and 200 μg/L.
219 The prefabricated coffee matrix was prepared as follows: 1.6 g CYS was added into
220 200 mL of freshly brewed coffee at 40 °C and dried by vacuum evaporation using a
221 rotary evaporation (BUCHI R-210, Switzerland). The residue was dissolved in
222 deionized 200 mL water. This procedure was repeat to minimize the level of 2-FFT in
223 the matrix. 1,2-Dichlorobenzene was prepared (1.5 μg in 10 μL of methanol) and was
224 added in 5 mL samples) as internal standard (Song, Zhang, Xiao, Niu, Hayat, & Eric,
225 2012; Zhang, Chen, Hayat, Duhoranimana, Zhang, Xia, et al., 2018). The standard
226 curve was presented by relative peak area (peak area of standard 2-FFT to that of
227 internal standard) to the concentration of standard 2-FFT ($y = 0.0055x + 0.0381, R^2 =$
228 0.998).
229 The quantification of all the volatiles (From Item 2.9) was based on the internal
230 standard method: the relative aroma concentration was calculated by comparing GC
231 peak areas of aromas with the area of internal standard (1,2-dichlorobenzene), using a
232 responding factor of 1 (Yang, Liu, Liu, Degn, Munchow, & Fisk, 2016; Zhang, et al.,
233 2018).
234 To calibrate the error from extraction procedure, samples of five milliliter were
235 placed into GC headspace sample vials (20 mL, Supelco, USA) with 1,2-
236 dichlorobenzene solution (1.5 μg in 10 μL of methanol) as the internal standard. The
237 vials were sealed by a screwed top with PTFE septum (Supelco, USA) and presented to

238 a 2-min incubation time under magnetic stirring at 40 °C (IKA RET control-visc, UK).
239 After incubation, the volatiles of vials in headspace were extracted by a
240 divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber of 75
241 μm film thickness with the manual SPME holder (Supelco, USA) at 40 °C for 10 min
242 (Mestdagh, Davidek, Chaumonteuil, Folmer, & Blank, 2014). The aromas were
243 desorbed for 3 min in the GC injector for 3 min at 250 °C in splitless mode and then
244 further separated by GC-MS.

245 The condition of GC-MS was set according to the library reports and modified
246 (Liu, Yang, Linforth, Fisk, & Yang, 2019; Mestdagh, Davidek, Chaumonteuil, Folmer,
247 & Blank, 2014; Sun, et al., 2018). A gas chromatograph coupled with a mass
248 spectrometer (TSQ Quantum XLS, Thermo Fisher Scientific Inc., USA) was equipped
249 with a capillary DB-WAX column (30 m × 0.25 mm × 0.25 μm film thickness, J & W
250 Scientific Inc., USA). The oven temperature was programmed as follows: 40 °C held
251 for 4 min, then increase to 160 °C at the rate of 3 °C/min, and rose to 240 °C at
252 20 °C/min, then held for 2 min; Energy voltage was 70 eV. Helium was used as carrier
253 gas at 1 mL/min. Injector temperature was 250 °C and splitless injector mode was used.
254 Selective Ion Monitoring (SIM) was used for quantification of 2-FFT from Item 2.3 to
255 2.8 and 114 *m/z* was used as the characteristic ion fragment (Sun, et al., 2018; Tominaga
256 & Dubourdieu, 2006). Full Scan mode was used (30 - 300 *m/z*) for the quantification of
257 general volatiles from Item 2.8. Samples were run in triplicate randomized order.

258 *2.2.8 Quantification of hydroxyhydroquinone in brewed coffee*

259 The quantification of HHQ was modified based on previous reports (Müller & 260 Hofmann, 2007; Sun, et al., 2018). Samples (20 mL) were subjected to liquid extraction 261 using ethyl acetate (20 mL × 2) followed by centrifugation at 4000 rpm for 4 min. After 262 supernatant collection, the solvent of the organic phase was removed by vacuum 263 evaporation at 20 mbar, then dissolved by methanol/water solution of 1 mL (1/1, *V/V*) 264 and filtered by polyvinylidene fluoride filters (PVDF, 0.45 Millipore). Aliquots of 10 265 μL were analyzed by an ultra-performance liquid chromatography tandem quadrupole 266 mass spectrometry (UPLC-TQ-MS, Waters, USA) with a reversed phase ACQUITY 267 UPLC® HSS T3 column (1.8 μm particle, 2.1 × 100 mm, Waters, USA) and quantified 268 by standard curve prepared by standard reference. Analytical conditions on the UPLC-269 MS were as follows: injection volume was 1 μL, flow rate was 0.3 mL/min, and the 270 column oven was set at 40 °C. Mobile phase A was 0.5% formic acid in methanol and 271 mobile phase B was 0.5% formic acid in water. The samples were analysed as follows: 272 phase A was held at 2% for 0.5 min, and then increased to 20% within 5.5 min. Phase 273 A was then increased to 100% in 4 min and held at 100% for 5.5 min. The mass spectra 274 were obtained by a Waters TQD system using the ionization conditions below: the 275 source block temperature and the desolvation temperature were 130 °C and 400 °C, 276 respectively; Capillary voltage was 3.47-3.50 kV, cone voltage was 20 V, and second 277 cone voltage was 3.00-3.17 V; Hexapole lens voltage was 0.30 V. The cone gas flow 278 was 50 L/h; the argon collision gas flow was 0.1 mL/min. The mass range was set as 279 *m*/*z* 100-1000 in the multiple reaction monitoring (MRM) mode to record the transition

280 from the negative pseudo-molecular ion $[M-H]^-$ to the fragment after collision-induced
281 dissociation (hydroxyhydroquinone *m/z* 125 → 75).
282 *2.2.9 Statistical Analysis*
283 Data were evaluated by analysis of variance using SPSS 19.0 (IBM, Chicago,
284 USA). Significant differences were evaluated by Duncan's multiple range test (DMRT,
285 post hoc test) to measure specific differences between pairs of means and the
286 significance level was set at 0.05. The correlation analysis between sensory analysis
287 and aroma compounds were analyzed by PLSR via Unscrambler 9.7 (CAMO ASA,
288 Norway).
289 **3. Results and discussion**
290 *3.1 2-furfurylthiol releasing mechanism and aroma releaser screen*
291 HHQ and free radical reaction path could lead to the reduction of 2-FFT in the
292 coffee brew (Müller & Hofmann, 2007, Blank, Pascual, Devaud, Fay et al., 2002). To
293 investigate the reaction between 2-FFT and HHQ, CYS, as a more active nucleophile
294 compared to 2-FFT, was added into a model experiment (Sun, et al., 2018). As CYS has
295 a greater affinity with HHQ, bound 2-FFT was released and the concentration of
296 measured 2-FFT increased by 83.2 μg/L (Fig. 1a). At the same time, the level of HHQ
297 decreased from 2.1 mg/L to 1.16 mg/L which might be induced from the generation of
298 new conjugates between HHQ and CYS.
299 HHQ can be easily oxidized to quinones by oxidants (Ingold, 1969). To further
300 investigate the intermediate reaction between HHQ and 2-FFT, the aroma binding
301 reactivity of quinones (OPH; the oxidation products of HHQ) was compared with HHQ.

302 In the model system, 2-FFT was incubated with OPH. It showed OPH was more
303 reactive and almost all 2-FFT reacted and was unavailable within 5 min (Fig. 1b-i). The
304 2-FFT incubated with HHQ, however, was still present at a concentration of 52.12 μg/L
305 at 5min, and it took 30 minutes to react fully. This indicates that OPH was more reactive
306 with 2-FFT than HHQ. To verify this, sodium sulfite (SS), which can reduce quinones
307 to phenols, was incubated with the oxidation products to reduce the interconversion of
308 HHQ to OPH (LuValle, 1952). It showed that 2-FFT was more stable and retained a
309 concentration of 45.21 μg/L after 40 min of incubation (Fig. 1b-i). This indicates that
310 the inhibition of the interaction between HHQ and its oxidation products will decrease
311 the 2-FFT binding reaction. Therefore, the oxidation products of HHQ might be the
312 intermediate reactant in the binding process between HHQ and 2-FFT (Fig. 1b-ii).

313 On the other hand, hydroxyl radicals generated from Fenton reaction have a
314 significant impact on coffee 2-FFT reduction. In Fenton reaction model, AA, as one of
315 the agents promoting the reaction, was used to reduce Fe (III) to Fe (II) in the model so
316 that the Fenton reaction can continue (Blank, et al., 2002). It was found that at low AA
317 concentrations (below 0.03 g/L), 2-FFT was decreased in concentration from 71.31 to
318 11.49 μg/L with increasing levels of AA (Fig. 1c). Fenton reaction can lead to 2-FFT
319 degradation (supplement Fig. A-i). With the increase of AA at a low level, Fenton
320 reaction scale would increase, which would lead to a further decrease of 2-FFT.
321 Furthermore, AA can also act as an antioxidant against the attack from free radicals,
322 such as hydroxyl radical, peroxyl and hydroperoxyl radicals (Niki, 1991). When AA
323 was present at higher concentrations (0.03 to 0.12 g/L), the level of 2-FFT increased to

324 51.55 μg/L with increasing levels of AA. This might be due to free radical quenching.
325 Since AA is a strong antioxidant and can scavenge hydroxy radicals, the increase of AA
326 makes the free radicals formed by the Fenton reaction be scavenged and lead to the
327 effective increase in 2-FFT concentration (chemical explanation shown in supplement
328 Fig. A-ii).

329 Based on above discussion, 2-FFT can be released by CYS and AA via HHQ path
330 and free radical inhibition respectively. By these aroma releasing mechanisms, five
331 ingredients were selected as potential 2-FFT release agents. Glutathione, as a mercaptan
332 compound, shows a nucleophile property as CYS; Sodium sulfite, as a strong reducing
333 agent, could reduce quinones to phenols (LuValle, 1952); Methionine has also been
334 reported to prevent melanin accumulation of fruit juices (Ali, Ahmad, Aman, et al.,
335 2018). Among the three sulfur compounds (Fig. 1d), methionine had no impact on the
336 level of 2-FFT, glutathione had a small 2-FFT releasing ability and CYS had the greatest
337 effect. Sodium sulfite, which is known for its reducing property, resulted in the second
338 highest 2-FFT concentration after addition at 1.5 g/L. Considering the reduction of
339 quinones from sodium sulfite in model system discussed above, the binding loss of 2-
340 FFT could be inhibited through the reduction of quinones. The highest releasing amount
341 of 2-FFT (up to 71.39 μg/L) was from the addition of CYS (1.5g/L), which provided
342 the evidence that the covalent binding was the dominant 2-FFT reduction approach as
343 our previous report (Sun, Yang, Liu, Linforth, Zhang, & Fisk, 2019). In brewed coffee,
344 2-FFT increased with the addition of AA (Fig. 1d), which induced from the free radical
345 scavenging effect of AA. Compared to other additives, AA presented a similar 2-FFT

346 releasing ability as sodium sulfite, 2-FFT increased from 12.30 to 32.59 μg/L and then
347 plateaus at concentrations above 0.05 g/L (Fig. 1d). However, considering the potential
348 dietary health risk, sodium sulfite was not recommended. Therefore, CYS in
349 combination with AA were selected as aroma releasers.

350 *3.2 Optimization the amount of aroma releasers used in coffee brew*

351 As the over addition of CYS would generate an undesirable smell, the off-flavor
352 threshold was detected using the forced-choice ascending method. The off-flavor group
353 threshold after CYS addition was 0.045 g/L (Table 1a), which was chosen as the optimal
354 used level.

355 AA was then added to the optimised used amount of CYS (0.045 g/L), at varying
356 levels to mitigate the loss of 2-FFT over storage. Coffee brew was stored with 5
357 different levels of AA and CYS and the resultant 2-FFT concentration was measured.
358 The concentration of 2-FFT increased from 32.60 to 43.84 μg/L with increasing amount
359 of AA from 0.03 to 0.04 g/L before incubation (Fig. 2). Additional AA above 0.04 g/L
360 did not result in a significant increase in 2-FFT before storage ($p < 0.05$). Over
361 incubation time and at low levels of AA addition, 2-FFT reduced below the level of
362 freshly brewed coffee, increasing amount of AA enhanced the level of 2-FFT released
363 over storage. The highest AA amount (0.07 g/L) had the highest 2-FFT level (31.28
364 μg/L) after incubation (30 min). However, the 2-FFT level with 0.05 g/L AA was 21.74
365 μg/L after 30min storage, which is similar to previous literature values of 20 μg/L in
366 fresh brewed coffee (marked by dotted line) (Mayer, Czerny, & Grosch, 2000; Sun, et
367 al., 2018). Therefore, the AA of 0.05 g/L was selected as the optimized amount.

368 *3.3 Impact of cysteine and ascorbic acid on coffee brew aroma profile over incubation*

369 During the incubation of coffee samples with CYS and AA for 30 min, sixty-two
370 volatile compounds were identified (Table 2). Among them, 17 different characteristic
371 aroma compounds were selected, as being well-known coffee aroma compounds that
372 have been previously published (Mayer, Czerny, & Grosch, 2000; Peter Semmelroch &
373 Grosch, 1995, 1996). After the addition of additives, sulfur compounds, pyrazines and
374 guaiacols significantly increased compared to the original fresh coffee brew (Fig. 3a).
375 Two sulfur-containing aroma compounds (2-FFT, and methyl furfuryl disulfide),
376 increased by 153% and 263% respectively. The reason can be proposed to the
377 competitive replacement and free radical scavenging action of CYS and AA (Charles-
378 Bernard, Roberts, & Kraehenbuehl, 2005; Sun, et al., 2018). These two volatile
379 compounds were known to contribute sulfurous and roasted aroma which would be lost
380 during coffee storage (Hofmann & Schieberle, 2002; Rowe, 2009). After 30 min of
381 incubation with CYS and AA (Fig. 3c), 91% of 2-FFT and methyl furfuryl disulfide
382 were retained compared to the fresh coffee brew. Dimethyl trisulfide was characterized
383 as an onion or smoky flavor (Flament & Bessière-Thomas, 2002), it increased by 270%
384 when CYS and AA were added (Fig. 3a) and its level stabilized after 30 min (97%).
385 Dimethyl trisulfide is a secondary production of dimethyl disulfide and they are both
386 generated from a strecker aldehyde, methional (Chan & Reineccius, 1994). Two
387 pyrazines also increased with the addition of CYS and AA, 3,5-dimethyl-2-
388 ethylpyrazine, which presents a roasted nutty aroma, increased by 140% and 2-ethyl-5-
389 methylpyrazine, which presents a roasted and ground smell, increased by 164%. These

390 two methylated pyrazines are generated from the Maillard reaction and the use of CYS
391 might enhance the formation of them (Weenen, Tjan, De Valois, Bouter, Pos, & Vonk,
392 1994). After storage, these two methylated pyrazines did not change with the addition
393 of stabilizers. 4-ethylguaiacol and 4-vinylguaiacol, as the degradation products of
394 ferulic acid (Schenker, Heinemann, Huber, Pompizzi, Perren, & Escher, 2002), could
395 present a characteristic smoky coffee odour (Flament & Bessière-Thomas, 2002). The
396 addition of CYS and AA increases 4-ethylguaiacol and 4-vinylguaiacol by 176% and
397 169%, respectively, and they were not lost during storage. A potential explanation is
398 that the mercapto group of CYS and the enol structure of AA can lead to a high reducing
399 property to protect the phenolic hydroxy of guaiacols from oxidation.

400 *3.4 Impact of cysteine and ascorbic acid on coffee brew sensory properties*

401 As the improvement of the coffee aroma quality during storage was the main aim
402 of this research, the sensory profile of the coffee brew prepared with CYS and AA
403 addition was evaluated and scored by quantitative descriptive analysis (QDA) (Stone,
404 Sidel, Oliver, Woolsey, & Singleton, 2008). The aroma characteristics of different
405 coffee samples were described as nutty, sulfurous, roasted, spicy, chocolaty, fruity,
406 flowery (Table 1b). Compared to freshly brewed coffee (Blank), the scores related to
407 nutty and roasted increased with the addition of CYS and AA. Specifically, nutty,
408 sulfurous and roasted aroma perception was the highest in the CYS & AA samples
409 compared to the Blank samples after 30min storage. This was correlated with the release
410 and stabilization of thiols and sulfur compounds by CYS and AA (Fig. 3). It is important
411 to note that the aromas not associated with the CYS and AA enhancement, such as fruity

412 and flowery aroma (Flament & Bessière-Thomas, 2002), were not enhanced, which
413 further supports the working hypothesis and proposed mode of action of CYS and AA.

414 The application of CYS and AA showed a significant enhancement on the overall
415 aroma intensity. After 30 min storage, the flavour intensity ratings of the blank coffee
416 reduced from 7.5 to 3.9, however with the addition of CYS and AA the coffee flavour
417 intensity was maintained at 7.1 (Table 1b). This again supports the working hypothesis
418 as the loss of these highly odour active compounds is likely to lead to flavour staling
419 (Hofmann & Schieberle, 2002; Mayer, Czerny, & Grosch, 2000). By selectively
420 stabilizing certain aroma compounds the flavour intensity of coffee brew was enhanced.
421 Preference was also evaluated, preference reduced from 7.4 to 3.5 in the blank coffee
422 brew over storage, however, the preference for CYS & AA samples was enhanced in
423 the fresh coffee brew (8.3) and maintained high (7.3) after 30 minutes storage (Table
424 1b).

425 *3.5 Correlation between sensory characteristics and aroma compounds*

426 Partial least squares regression (PLSR) analysis was conducted to investigate the
427 correlation between aroma compounds analyzed from SPME-GC-MS and sensory
428 characteristics from panelists. In the correlation loading plot (Fig. 4), the sixty-two
429 aroma compounds detected by SPME-GC-MS (Table 2). Concentration data was in
430 supplement Table A) was set as X variables and nine sensory characteristics collected
431 from the quantitative descriptive analysis test (Table 1 b) was set as Y variables. This
432 model contained three significant principle components as PC1, PC2 and PC3. For PC1

433 and PC2, 62% of X variables and 77% of Y variables were explained. The loading plot
434 of PC2 versus PC3 was not presented since no additional information was obtained.
435 All X variables and most of the Y variables were presented between the inner and
436 outer ellipse, which indicated that the correlation between aroma characterisitics and
437 volatiles could be well explained. When considering the sensory characteristics (Table
438 1b), nutty, sulfurous and chocolaty were correlated with aroma intensity and preference
439 and are shown in the upper-right part of the bi-plot. Flowery and spicy were also
440 correlated and are shown in the lower-right part of the bi-plot. Roasted not correlated
441 with the other aroma descriptors and is shown on the left of the bi-plot. Twenty-nine
442 aroma compounds, which were marked by blue hollow circles, showed significant
443 correlation with sensory characteristics.
444 Nutty, sulfurous and chocolaty were correlated with pyrazines, sulfurous
445 compounds and aldehydes (yellow circle in Fig. 4). PLSR1 regression showed in more
446 detail that nutty was positive correlated to 2,6-dimethylpyrazine (26, the number is
447 corresponding to the compounds number in Table 2, Figure 4 and Supplimentary Figure
448 B), 2-propylpyrazine (33), 2,6-diethylpyrazine (35) and, 2,4,5-trimethylthiazole (20)
449 (filled by slash, supplement Fig. B I). Sulfurous aroma is important for freshness
450 perception in coffee (Hofmann & Schieberle, 2002; Marin, Požrl, Zlatić, & Plestenjak,
451 2008) and was positively correlated with 2-FFT (34), methyl furfuryl disulfide (54),
452 dimethytrisulfide (29) and 2,4,5-trimethylthiazole (20) (supplement Fig. B II) and
453 negatively correlated with furfural (37) and furfuryl propinonate (47). Chocolaty was
454 positively correlated with 2,6-dimethylpyrazine (26), 2-FFT (34), methyl furfuryl

455 disulfide (54) and 4-vinylguaical (61) (supplement Fig. B III). Roasted is is positively
456 correlated with three pyrazines, 2-ethyl-3-methylpyrazine (32), 2,6-diethylpyrazine (35)
457 and 3-ethyl-2,5-dimethylpyrazine (36), two phenols such as 4-vinylguaiacol (61) and
458 4-ethylguaiacol (62), and furfuryl methyl sulfide (39). 2-methylfuran (2), 2-
459 methylbutanal (3) and several ketones, aldehydes and esters were negatively correlated
460 to roasted (Fig. 4 and supplement Fig. B IV). Flowery and spicy, which were not
461 modified by CYS and AA, are associated with diones and pyrroles (green circle) in Fig.
462 4, further details are shown in the supplementary data (Fig. B V-VII). Overall, the
463 addition of CYS and AA stabilized roasted, nutty, chocolaty and sulfurous which played
464 an important role in maintaining coffee aroma intensity.

465 **4. Conclusion**

466 In coffee brew, a key aroma compound 2-FFT is readily lost during storage as it
467 reacts with other coffee compounds. We showed that the addition of CYS decreased the
468 level of free HHQ and directly led to a release of 2-FFT that was previously bound.
469 Furthermore, we showed a greater binding reactivity of HHQ oxidation products, such
470 as quinones, this suggests that the oxidation products might be the direct binding
471 reactant of 2-FFT, and sodium sulfite was shown to inhibit this binding. In a model
472 system, low levels of AA (0.03 g/L) intensified the loss of 2-FFT, and high levels of AA
473 (0.04-0.07 g/L) stabilized 2-FFT. Building on this, CYS and AA were proposed as
474 feasible releasers of 2-FFT for coffee applications. The concentration of CYS and AA
475 was optimised to minimise off-flavor whilst enhancing 2-FFT release; 0.045 g/L CYS
476 and 0.05 g/L AA were shown to release and stabilize 2-FFT over 20 μg/L for 30 min.

477 Furthermore, the addition of CYS and AA enhances the amount of pyrazines, guaiacols
478 and sulfur compounds in fresh coffee. A correlation study via partial least squares
479 regression analysis showed that nutty, sulfurous, and chocolaty was positivity
480 correlated with some of the pyrazines, thiols, phenols and sulfides ($p < 0.05$). While
481 flowery and fruity was positively correlated with the esters, aldehydes and ketones.
482 Sensory testing further showed an enhancement in nutty, sulfury and roasted aroma
483 attributes, a direct increase in aroma intensity and sensory evaluated preference over
484 shelf life.

485 This work showed the mechanism of 2-FFT loss via the inhibition of the 2-FFT
486 degradation pathway and provides new solutions to improve the rapid loss of sulfury-
487 roasted aroma in liquid coffee. Commercially, this could be used to stabilise the flavour
488 of liquid coffees.
489

Acknowledgements

This research was financially supported in part by National Key R&D Program of China 2017YFD0400105, National First-class Discipline Program of Food Science and Technology JUFSTR20180204 and ‘‘Collaborative innovation center of food safety and quality control in Jiangsu Province’’. The authors greatly appreciate the support by the Biotechnology and Biological Sciences Research Council, United Kingdom [grant number BB/R01325X/1]. Professors Xiaoming Zhang and Ian D. Fisk are acknowledged as dual corresponding authors

Conflict of interest

The authors declare that they have no competing financial interests.

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