

1 **Aroma binding and stability in brewed coffee: a case study of 2-furfurylthiol**

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17 **Abstract:** The aroma stability of fresh coffee brew was investigated during storage over  
18 60 minutes, there was a substantial reduction in available 2-furfurylthiol (2-FFT) (84%),  
19 methanethiol (72%), 3-methyl-1H-pyrole (68%) and an increase of 2-pentylfuran  
20 (65%). It is proposed that 2-FFT was reduced through reversible chemical binding and  
21 irreversible losses. Bound 2-FFT was released after cysteine addition, thereby  
22 demonstrating that a reversible binding reaction was the dominant mechanism of 2-FFT  
23 loss in natural coffee brew. The reduction in available 2-FFT was investigated at  
24 different pH and temperatures. At high pH, the reversible binding of 2-FFT was shown  
25 to protect 2-FFT from irreversible losses, while irreversible losses led to the reduction  
26 of total 2-FFT at low pH. A model reaction system was developed and a potential  
27 conjugate, hydroxyhydroquinone, was reacted with 2-FFT. Hydroxyhydroquinone also  
28 showed 2-FFT was released after cysteine addition at high pH.

29 **Key words:** coffee brew aroma stability; 2-furfurylthiol (2-FFT); reversible and  
30 irreversible degradation; binding site stability

31 **Chemical compounds studies in article:** 2-furfurylthiol (PubChem CID 7363); L-  
32 cysteine (PubChem CID 5862); hydroxyhydroquinone (PubChem CID 10787); Sodium  
33 dihydrogen phosphate (PubChem CID: 23672064); disodium hydrogen phosphate  
34 (PubChem CID: 24203); hydrochloric acid (PubChem CID: 313); sodium hydroxide  
35 (PubChem CID: 14798); 3-heptanone (PubChem CID: 7802)

36

## 37 1. Introduction

38 After coffee roasting, furans, pyrazines, thiols, aldehydes and many other volatiles  
39 are present in the headspace (Yang et al., 2016) and together form the complex aroma  
40 profile of coffee brew (Sammelroch & Grosch, 1995). However, some of these coffee  
41 aroma compounds are unstable during storage of roasted coffee and coffee brew  
42 (Dulsat-Serra, Quintanilla-Casas, & Vichi, 2016). This aroma deterioration results from  
43 the interaction with the matrix (Fisk, Boyer, & Linforth, 2012; Yu et al., 2012) and  
44 aroma degradation, and is termed aroma staling (Hofmann & Schieberle, 2002, 2004;  
45 Müller & Hofmann, 2007).

46 2-Furfurylthiol (2-FFT), as a sulfur compound in coffee, has been established as  
47 one of the key aromas that contribute to the characteristic flavour of coffee based on  
48 sensory studies and model dilute experiments (Blank, Sen, & Grosch, 1992; Hofmann  
49 & Schieberle, 2002; Semmelroch & Grosch, 1995). However, 2-FFT rapidly reduces  
50 during coffee brew processing or storage due to these staling reactions. This loss could  
51 cause a significant reduction of sulfury-roasty aroma and is partially responsible for the  
52 inferior sensory quality of aged coffee brews (Hofmann, Czerny, & Schieberle, 2001;  
53 Mayer, Czerny, & Grosch, 2000; Semmelroch & Grosch, 1996).

54 The reduction of available 2-FFT during coffee staling can be divided into  
55 reversible and irreversible staling events (Charles-Bernard, Kraehenbuehl, Rytz, &  
56 Roberts, 2005; Guichard, 2002). The irreversible losses of 2-FFT are presumed to be  
57 due to physical diffusion/volatile loss and chemical degradation reactions, such as  
58 polymerization or oxidation (Blank et al., 2002; Charles-Bernard, Kraehenbuehl, et al.,

59 2005). This irreversible fraction is very hard to regenerate. Reversible losses are  
60 believed to be mainly through covalent bonding to non-volatile components in the  
61 coffee matrix, it is proposed that this 2-FFT lost due to reversible reactions could be  
62 subsequently released again by cysteine addition (Müller & Hofmann, 2007; Mestdagh,  
63 Davidek, Chaumonteuil, Folmer, & Blank, 2014; Sun et al., 2018).

64 Previous studies have shown that the thiol group of 2-FFT is a good nucleophile  
65 and could be involved in nucleophilic and radical reactions (Rowe, 2009). Through the  
66 binding reaction, 2-FFT could be reversibly bound to conjugates in coffee brew, such  
67 as 1, 4-bis (5-amino-5-carboxy-1-pentyl) pyrazinium radical cation (CROSSPY) that is  
68 found in coffee melanoidins (Hofmann & Schieberle, 2002; Tominaga, Blanchard,  
69 Darriet, & Dubourdieu, 2000). More recent studies have reported that  
70 hydroxyhydroquinone (HHQ), one of the chlorogenic acid degradation products, was  
71 the dominant conjugate to bind 2-FFT (Müller & Hofmann, 2007). In this binding  
72 reaction, 2-FFT is bound by HHQ through the reactive quinone converted from HHQ,  
73 leading to a rapid reduction of 2-FFT (Fig. 1) (Müller, Hemmersbach, van't Slo, &  
74 Hofmann, 2006; Müller & Hofmann, 2007). Hofmann and Schieberle showed that this  
75 covalent binding could be established within 15 min (Hofmann & Schieberle, 2002)

76 Cysteine has been shown previously to release 2-FFT that is bound by coffee  
77 matrix (Darriet, Tominaga, Lavigne, Boidron, & Dubourdieu, 1995; Mestdagh et al.,  
78 2014). After cysteine addition, the bound form of 2-FFT is competitively replaced by  
79 cysteine due to its mercapto structure and high reducing properties. High cysteine  
80 concentration could also prevent 2-FFT from forming dimers (Rowe, 2009). Cysteine

81 addition has also been shown to reversibly release bound 2-FFT in coffee brew. This  
82 has been used to determinate the total: bound 2-FFT in coffee brews. Hereinafter “total  
83 2-FFT” refers to the whole available 2-FFT in the coffee brew at any time point,  
84 including both free 2-FFT and reversibly bound 2-FFT. “Free 2-FFT” means the 2-FFT  
85 fraction that exists in free form in a coffee brew (Sun et al., 2018).

86 pH is important for 2-FFT formation and loss in coffee brew. High pH conditions  
87 favor the formation of 2-FFT during coffee roasting and also increased 2-FFT loss  
88 during heating in model systems (Hofmann & Schieberle, 1998; Kumazawa & Masuda,  
89 2003). However, the impact of pH on coffee aroma binding in coffee brews, and the  
90 relative understanding of free: bound 2-FFT in the coffee brew and their relative  
91 stabilities are not well understood and is therefore the focus of this study.

92 In this study, coffee aroma staling was evaluated and the mechanism behind 2-FFT  
93 loss investigated. The stability of free versus total 2-FFT was evaluated. Reversibly  
94 bound 2-FFT was released by the addition of cysteine enabling the calculation of the  
95 reversibly bound and irreversibly lost 2-FFT fractions with the further calculation of  
96 losses of 2FFT through volatilization. The effects of pH and temperature on 2-FFT  
97 binding capacity was evaluated for coffee brew. To elucidate the pH effect on potential  
98 conjugate, HHQ, a model system was developed to explain the interaction of HHQ and  
99 2-FFT with or without cysteine addition. Aroma loss through binding reactions is a  
100 common costly problem for the soluble coffee industry, Robusta coffee is commonly  
101 used in this field, therefore Robusta coffee was selected as the target for this study.

## 102 2. Materials and methods

103 *2.1 Materials and reagents*

104 2-furfurylthiol (purity  $\geq$  98%), hydroxyhydroquinone (purity  $\geq$  99%),  
105 homogenous series of *n*-alkanes (C6-C26) standard were purchase from Sigma-Aldrich  
106 company (Poole, UK). Sodium dihydrogen phosphate (purity  $\geq$  99%), disodium  
107 hydrogen phosphate (purity  $\geq$  99%), sodium hydroxide (purity  $\geq$  97%), methanol  
108 (purity  $\geq$  99%), concentrated hydrochloric acid (purity  $\geq$  36.5%), L-cysteine (purity  $\geq$   
109 98%), 3-heptanone (purity  $\geq$  99%) were purchased from Acros Organic company (New  
110 Jersey, USA). Robusta washed coffee beans from Vietnam were purchased from  
111 Pennine Tea and Coffee Company (Halifax, UK).

112 *2.2 Coffee sample preparation*

113 Green Robusta washed coffee beans (250 g) were roasted by a convection oven  
114 (Mono Equipment, Swansea, UK). Before roasting, the oven was pre-heated for 5 min  
115 to reach 225 °C. The coffee beans were roasted for 15 min at  $225 \pm 2$  °C and, after  
116 cooling to 20 °C by convective cold air (around 10 °C cooling for 5 min), were ground  
117 using a coffee grinder for 11 seconds (KG 49, Delonghi, Australia). The ground coffee  
118 was screened by a metal sieve (700  $\mu$ m, Endecotts, Essex, UK) to control particle size.  
119 The color of roasted ground coffee was analyzed by an UltraScan Pro (Hunterlab, US)  
120 in a 1 cm Petri plate. The result was:  $L^*$  (lightness) =  $41 \pm 0.3$ ,  $a^*$  (red, green) =  $3.74 \pm$   
121  $0.2$ ,  $b^*$  (yellow, blue) =  $3.81 \pm 0.3$ . The  $L^*$ ,  $a^*$  and  $b^*$  values were in accordance with  
122 the optimal roasting degree of medium roasting (Mendes, de Menezes, Aparecida, &  
123 Da Silva, 2001). All ground samples were stored at -80 °C under nitrogen gas. Before  
124 brewing, ground samples were defrosted at 15 °C for 15 min.

125 The ground coffee (9 g) was brewed using a French press (3 Cup Black Cafetière,  
126 Argos, UK). Before brewing, the French press was rinsed by deionized water (around  
127 92 °C) to preheated it for 10 seconds. Then the ground coffee (9 g) was mixed by  
128 deionized water (180 mL, 92 °C) in the French press coffee maker. The plunger was  
129 pressed down until the filter touched the coffee liquid surface and stood for 4 min before  
130 depressing the plunger. The resulting fresh coffee liquid was shaken gently in French  
131 press for 5 seconds to make coffee brew uniform.

132 In order to minimize the volume of headspace and exclude the aroma physical  
133 diffusion, the coffee brew was poured from French press to fill full an amber vial of 40  
134 mL which was sealed by screw top with PTFE-lined silicone septa (Supelco, Sigma  
135 Aldrich, UK). The sealed amber vials with fresh coffee were cooled to 40 °C by water  
136 bath and following experiments were started from these “sealed amber-vial samples”  
137 respectively.

### 138 2.3 Coffee aroma stability

139 The sealed amber-vial samples were stored in a water bath (FB60307, Fisher  
140 Brand, UK) up to 60 min (0, 15, 30, 45, 60 min) at 40 °C prior to solid-phase  
141 microextraction-gas chromatography mass spectrometry (SPME-GC-MS).

### 142 2.4 Effect of storage time on *free and total* 2-furfurylthiol concentration *under open-* 143 *vial condition*

144 Five milliliter of fresh coffee brew was transferred from the sealed amber-vial  
145 samples (prepared from item 2.2) to GC headspace vials (20 mL, 22.5 mm × 75.5 mm,  
146 Sigma-Aldrich, UK) by pipette (Argos, UK). The GC headspace vials were left open

147 (open-vial condition) to allow aroma volatilization so that the physical diffusion loss  
148 was evaluated. The headspace vials were stored for up to 60 min at 40 °C in a water  
149 bath before sealing for free and total 2-FFT SPME-GC-MS analysis.

#### 150 2.5 Effect of pH and storage temperature on free and total 2-FFT in coffee brew

151 The sealed amber-vial samples were adjusted to pH 3 to 9 with 1 M hydrochloric  
152 acid solution and 1 M sodium hydroxide solution. Samples were stored at 20, 55 or  
153 90 °C in a water bath for 1 h respectively. After cooling down to 40 °C by water bath,  
154 the free 2-FFT concentration of samples was analyzed by SPME-GC-MS. Another  
155 aliquot of 40 mL amber-vial sample was subjected to the same pH incubation (without  
156 different temperature storage). Then the total 2-FFT concentration was analyzed by  
157 SPME-GC-MS.

158 To further study the pH effect on 2-FFT binding and release, additional  
159 experiments were also performed:

160 **Cysteine addition after pH incubation:** The sealed amber-vial samples (40 mL) were  
161 adjusted to pH 3 or 9, and stored for 1 h in water bath at 55 °C. After that, the pH of  
162 samples was adjusted back to 6 prior to total 2-FFT analysis by SPME-GC-MS.

163 **Cysteine addition before pH incubation:** Cysteine of 0.64 g was added into the sealed  
164 amber-vial samples (40 mL) after cooling down to 40 °C by water bath (to prevent  
165 cysteine from undergoing Maillard reaction at high temperature) (Sun et al., 2018) and  
166 stirred at 1000 rpm for 5 min on a magnetic stirrer (IKA RET control-visc, UK). The  
167 pH of samples was adjusted to 3 and 9. After 1 h storage in a water bath at 55 °C, the  
168 total 2-FFT was analyzed via SPME-GC-MS.



169 *2.6 Effect of pH on free and total 2-FFT in model system*

170 The buffer solution was prepared using disodium phosphate solution (0.2 M),  
171 sodium dihydrogen phosphate solution (0.2 M) and sodium hydroxide solution (0.1 M)  
172 and hydrochloric acid solution (0.1 M) to adjust the pH to 3 or 9. An aqueous solution  
173 of hydroxyhydroquinone (HHQ) (0.004 g HHQ diluted in 30 mL Milipore water) was  
174 added (300  $\mu$ L) into phosphate buffer solution (40 mL) and placed in amber vials (40  
175 mL), the samples was then stored for 1 h in a water bath at 40 °C to allow incubation  
176 (Müller & Hofmann, 2007). 2-FFT (36  $\mu$ g in 31.7  $\mu$ L of methanol) was added into HHQ  
177 buffer solution. 2-FFT was reacted with HHQ for 1 h at 40 °C prior, to evaluate free  
178 and total 2-FFT by SPME-GC-MS.

179 *2.7 Quantification of volatile compounds*

180 The quantification of 2-FFT was carried out using the internal standard  
181 quantification method (Sun et al., 2018). 3-Heptanone (4.1  $\mu$ g in 5  $\mu$ L of methanol) was  
182 added into calibration solution as an internal standard (IS) to accommodate for  
183 instrument drift. For coffee brew, the calibration curve was established by adding 2-  
184 FFT (0, 1.8, 3.6, 7.2, 14.4  $\mu$ g in 31.7  $\mu$ L of methanol) into a prefabricated coffee model  
185 which had a similar matrix to coffee brew. To prepare this coffee model, cysteine (1.6  
186 g) was presented into fresh coffee brew (200 mL) at 40 °C. The coffee brew was dried  
187 by rotary vacuum evaporation at 40 °C. The dried sample was then dissolved in 200  
188 mL of ultra-pure water. This part of experiment (from adding cysteine to samples  
189 dissolved into water) was repeated once more to release maximum 2-FFT from the  
190 coffee brew (Sun, et al., 2018). For the model experiment, the calibration curve was

191 established by adding 2-FFT (0, 9, 18, 36, 44  $\mu\text{g}$  in 31.7  $\mu\text{L}$  of methanol) into the  
192 phosphate buffer solution. The concentration of 2-furfurylthiol could be calculated by  
193 the calibration curve made by relative peak area (peak area of standard 2-FFT to that of  
194 internal standard) to the concentration of standard 2-FFT ( $y = 0.0055x + 0.0381$ ,  $R^2 =$   
195 0.998) .

196 Approximate quantification of all volatiles for the aroma stability study (Item 2.3)  
197 were calculated by comparing the GC peak areas of volatiles with the peak area of 3-  
198 heptanone internal standard, using a response factor of 1 to give relative concentrations  
199 (Liu, Yang, Linforth, Fisk, & Yang, 2019). The percentage of volatile was calculated by  
200 the comparison of their relative concentration after storage to their initial relative  
201 concentration in fresh coffee. All samples were analyzed in one run in randomized order.

## 202 2.8 SPME-GC-MS

203 Five milliliters of samples were placed into GC headspace vials (20 mL, 22.5 mm  
204  $\times$  75.5 mm, Sigma-Aldrich, UK) from the amber vials by pipette. 3-heptanone (4.09  $\mu\text{g}$   
205 in 5  $\mu\text{L}$  of methanol) (Caporaso, Whitworth, Cui, & Fisk, 2018; Liu et al., 2019) was  
206 added into the samples as the internal standard before vials were sealed by screw top  
207 with PTFE-lined silicone septa. For total 2-FFT analysis, cysteine powder was added  
208 to each vial before closure at 16 g/L (Sun et al., 2018) so that the bound form of 2-FFT  
209 was released to the free form. With cysteine, the free 2-FFT currently detected by  
210 SPME-GC-MS was the amount of total 2-FFT. Aroma analysis for free 2-FFT in coffee  
211 brew had no cysteine addition.

212 The analysis was carried out using a gas chromatography coupled with the Single-

213 Quadrupole Mass Spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK). A  
214 50/30  $\mu\text{m}$  DVB/CAR/PDMS SPME fiber (Supelco, Sigma Aldrich, UK) was used to  
215 extract volatile aroma compounds from the samples headspace. Samples were  
216 incubated at 40  $^{\circ}\text{C}$  for 2 min. The SPME extraction procedure was presented at 40  $^{\circ}\text{C}$   
217 for 10 min. The fiber desorption was at 250  $^{\circ}\text{C}$  for 3 min in the injector and then  
218 analyzed by GC-MS.

219 A ZB-WAX column (30 m  $\times$  0.25 mm I.D., 1  $\mu\text{m}$  film thickness; Phenomenex Inc,  
220 Macclesfield, UK) was used to separate constituents. Analytical GC conditions in GC-  
221 MS were as follows: Helium was used as carrier gas (1 mL/min). Injector temperature  
222 was 250  $^{\circ}\text{C}$ . Splitless injector mode was used. The oven temperature program was held  
223 at 40  $^{\circ}\text{C}$  for 5 min, then raised at a rate of 3  $^{\circ}\text{C}$  /min to 160  $^{\circ}\text{C}$ . After that, oven  
224 temperature was raised at 20  $^{\circ}\text{C}$  /min to 240  $^{\circ}\text{C}$  and held for 2 min; Energy voltage was  
225 70 eV. Single ion monitoring (SIM) and Scan mode was used (30 – 300 m/z). Samples  
226 were run in triplicate randomized order.

227 2-FFT was identified by comparing the detected 2-FFT mass spectra and linear  
228 retention indices (LRI) with 2-FFT standard mass spectra and LRI value from reference  
229 data libraries (NIST 11 and WILEY 07 databases). 114 m/z was used as the  
230 characteristic ion for 2-FFT quantification (Mestdagh et al., 2014; Tominaga &  
231 Dubourdieu, 2006). The identification of other volatiles was identified by comparing  
232 the detected volatiles mass spectra and LRI value with the mass spectra and LRI value  
233 from data libraries (NIST 11 and WILEY 07 databases). The linear retention indices  
234 values were calculated with homogenous series of n-alkanes (C6-C26) standard in the

235 same GC conditions.

## 236 2.9 Statistical Analysis

237 Data were presented as mean values  $\pm$  standard deviation. Statistical analysis was  
238 conducted by SPSS 19.0 (SPSS Inc., Chicago, USA). Duncan's multiple range tests  
239 was used, and  $p < 0.05$  was considered as significant. All samples were measured in  
240 triplicate.

## 241 3. Results and discussion

### 242 3.1 Coffee aroma stability

243 To identify which coffee volatiles are directly impacted by chemical changes over  
244 storage, samples were stored in fully filled vials. This allowed the investigation of  
245 storage time on aroma, while excluding the effects of physical diffusion and losses into  
246 the headspace. Eighty-nine volatile aroma compounds were detected in the fresh coffee  
247 brew and after 1 h storage at 40 °C, there were substantial changes over storage time in  
248 the aroma concentration of four aroma compounds, shown in Fig. 2 A to D. 85 of the  
249 volatile aroma compounds did not change their relative headspace concentration by  
250 more than 50%. The headspace relative concentration of 2-furfurylthiol, methanethiol  
251 and 3-methyl-1H-pyrrole decreased by 84%, 72%, 68% respectively; 2-pentylfuran  
252 increased to 165% compared to its relative concentration in coffee brew before storage.  
253 Of all the compounds evaluated, 2-FFT showed the fastest decrease, with a 68%  
254 reduction in free 2-FFT within 15 min. It should also be noted that of the four  
255 compounds that changed, 2-FFT has the highest odour activity value (OAV) and highest  
256 flavor dilution factor (FD factor) by aroma extract dilution analysis (Semmelroch &

257 Grosch, 1995; Vermeulen, Gijs, & Collin, 2005), and a sulfury-roasty aroma, which is  
258 highly characteristic of coffee aroma (Akiyama et al., 2008; Dulsat-Serra et al., 2016).  
259 Therefore, 2-FFT was selected as the key instable aroma compound for further studies.

### 260 3.2 Effect of storage time on reversible and irreversible binding of 2-FFT under open- 261 vial condition

262 Total available 2-FFT can be lost by irreversible losses and reversible binding  
263 reactions (Charles-Bernard, Kraehenbuehl, et al., 2005; Guichard, 2002). These two  
264 losses affect the total and potential for free 2-FFT in coffee. As the total 2-FFT includes  
265 free 2-FFT and reversibly bound 2-FFT, the difference between the total 2-FFT amount  
266 and free 2-FFT amount is defined as “reversibly bound 2-FFT”; On the other hand,  
267 irreversible losses occurred and increased during coffee storage. So, the difference of  
268 total 2-FFT between original and stored coffee is defined as “irreversibly lost 2-FFT”.  
269 Thus, the total and free 2-FFT of the stored coffee were determined to investigate the  
270 extent of reversibly bound and irreversibly lost 2-FFT during storage. Over 1h storage,  
271 the concentration of both total and free 2-FFT decreased significantly (Table 1). The  
272 biggest change happened within the first 30 minutes and then plateaued. Total 2-FFT  
273 decreased from 153 to 138  $\mu\text{g/L}$  (10% loss) ( $p < 0.05$ ), and free 2-FFT decreased from  
274 3.4 to 0.4  $\mu\text{g/L}$  (88% loss) over 1 h storage ( $p < 0.05$ ). The total 2-FFT concentration was  
275 much higher than free 2-FFT concentration, indicating that a large amount of 2-FFT  
276 was reversibly bound to coffee components within the fresh coffee brew.

277 Using the free and total 2-FFT amounts, the reversibly bound and irreversibly lost  
278 2-FFT amount could be calculated using the equation below. After a defined period of

279 storage (X min).

280  $Reversibly\ bound\ 2\text{-FFT} = Total\ X - Free\ X$

281  $Irreversibly\ lost\ 2\text{-FFT} = Total\ O - Total\ X$

282 *X*: the concentration of aroma in coffee brew stored at time X min;

283 *O*: the concentration of aroma in original fresh coffee brew at time 0 min.

284 Reversibly lost 2-FFT and irreversibly lost 2-FFT are presented in Fig. 3. Over the  
285 1 h storage period, the proportion of reversibly bound 2-FFT reduced from 149.6 to  
286 137.1 µg/L). The irreversibly lost 2-FFT increased from 0 to 15.5 µg/L. These low  
287 (~10%) losses suggest that the volatilization and irreversible chemical degradation have  
288 a limited effect on 2-FFT loss compared to the predominant reversible binding in  
289 natural coffee brew (~pH 6.2). Due to this, in natural coffee (~pH 6.2), reversible loss  
290 is proposed to be the main reason for the loss of free 2-FFT, which is expected to play  
291 a significant role in the loss of the characteristic aroma of coffee during staling  
292 (Hofmann & Schieberle, 2002; Mayer & Grosch, 2001).

293 *3.3 The effect of pH and storage temperature on free 2-FFT concentration in coffee*  
294 *brew and the model system*

295 Coffee brew samples were stored at different pH and temperatures to investigate  
296 the impact on free 2-FFT stability. Both pH and temperature (Fig. 4 A) had a significant  
297 impact on free 2-FFT concentration. After 1 h incubation at elevated temperatures, free  
298 2-FFT concentration decreased significantly (2.7 to 0.4 µg/L at 20 °C; 1.9 to 0.5 µg/L  
299 at 55 °C; 1.6 to 0.5 µg/L at 90 °C), the loss of free 2-FFT was greatest at highest pH  
300 values at all temperature (Fig. 4 A). This resulted in almost all free 2-FFT being

301 reversibly bound by the coffee matrix at pH 6-9. The same trend was also found in the  
302 model system (Fig. 4 B) where the concentration of free 2-FFT decreased from 383 to  
303 0.02 µg/L when the pH of buffer solution was increased from 3 to 9.

304 This pH sensitivity can be explained by the impact on quinone. As discussed  
305 previously, during the reversible binding reaction between 2-FFT and HHQ, the  
306 reactive quinone is formed from HHQ first, then it reacts with 2-FFT (Fig. 1) (Müller  
307 & Hofmann, 2007). The highly reactive quinone is unstable at low pH due to its  
308 carbonyl property (Li & Chen, 2005). So, under low pH conditions, the reversible  
309 binding reactions are inhibited resulting in a greater concentration of free 2-FFT at low  
310 pH as shown in Fig. 4 A and B. The inverse of this means that at high pH the conversion  
311 of quinone is not be inhibited and more free 2-FFT is reversibly bound.

312 The 2-FFT in coffee brew stored at 20 °C had the highest free 2-FFT concentration  
313 (Fig. 3 A), while at 90 °C there was less free 2-FFT compared to samples stored at 20  
314 and 55 °C. This result suggests that the high temperature may increase the efficiency of  
315 the reversible binding reaction leading to more efficient 2-FFT binding.

#### 316 3.4. The effect of pH on total 2-FFT concentration in coffee brew and model system

317 pH also impacted the total 2-FFT level. After 1 h storage, total 2-FFT showed a  
318 significant increase when the incubation pH was increased (Fig. 4 C), increasing from  
319 2.6 to 159 µg/L. In the model system, the similar increase was found, but to a lesser  
320 extent (351 to 524 µg/L) (Fig. 4 D).

321 To study the reason for the low level of total 2-FFT at low pH and high level at  
322 high pH value, 2-FFT releasing ability of cysteine was further studied as cysteine might

323 be sensitive to pH. Before cysteine addition, samples were incubated at different pH  
324 and were adjusted back to pH 6, as cysteine has previously been shown to effectively  
325 release reversibly bound 2-FFT at pH 6 (Mestdagh et al., 2014; Sun et al., 2018). After  
326 control of the pH, the result still showed a low total 2-FFT level when stored at low pH  
327 and high total 2-FFT level when stored at high pH (Fig. 5: Pre pH incubation). The total  
328 2-FFT at pH 9 (105 µg/L) was over twice as the amount retained when the coffee brew  
329 was stored at pH 3 (50 µg/L), suggesting that the 2-FFT releasing ability by cysteine  
330 was not susceptible to pH.

331 With the addition of cysteine, total 2-FFT at pH 3 was much lower than pH 9 as  
332 shown in Fig. 4 C. This low amount of total 2-FFT might be a result of irreversible  
333 losses as the reversible binding is inhibited at low pH (Discussed in Item 3.3). To further  
334 verify this irreversible loss effect on total 2-FFT, cysteine was added into coffee brew  
335 before pH incubation (Fig. 5.). After pH incubation, total 2-FFT under both pH 3 and 9  
336 incubation presented a much lower level (56.8 µg/L and 49.9 µg/L). This result  
337 indicated that when the bound 2-FFT was released out of the “protection” of conjugates  
338 (such as HHQ) prior to incubation, the total 2-FFT was equally lost by the irreversible  
339 losses occurring during incubation. Suggesting that 2-FFT is protected by reversible  
340 binding during incubation.

341 It should also be noted that the total 2-FFT concentration in the model system at  
342 pH 3 (Fig. 4 D) was not degraded as much as the total 2-FFT in the coffee brew (Fig. 4  
343 C). Suggesting other effects caused by the matrix difference between the coffee and the  
344 model system. In coffee brew, the matrix is much more complex and would increase



345 irreversible losses through other mechanisms such as radical delivery from Fenton  
346 reaction (Charles-Bernard, Roberts, & Kraehenbuehl, 2005). This suggests that  
347 additional coffee non-volatiles do contribute to 2-FFT irreversible losses (Charles-  
348 Bernard, Kraehenbuehl, et al., 2005; Charles-Bernard, Roberts, et al., 2005).

#### 349 4. Conclusion

350 This study identified four unstable aroma compounds in coffee brew, the  
351 availability of which changed significantly over a 1h holding period, these were 2-  
352 furfurylthiol, methanethiol, 3-methyl-1-pyrole and 2-pentylfuran. 2-furfurylthiol  
353 suffered the greatest losses and was selected for further investigation. It was shown that  
354 reversible binding with HHQ was the dominant reason for 2-furfurylthiol staling in  
355 natural coffee. To further explain this reversible binding reaction, 2-FFT binding was  
356 studied and showed that at low pH, the 2-FFT binding reaction to hydroxyhydroquinone  
357 (HHQ) is inhibited and the availability of free 2-FFT level increased compared to free  
358 2-FFT at higher pH values. This work also showed that without the protection of  
359 conjugates (reversibly bound to HHQ) in coffee brew, free 2-furfurylthiol could be  
360 irreversibly lost.

361 The key finding that high levels of reversible binding of 2-FFT occurs during  
362 storage at an optimal pH and this stabilizes key aroma compounds from irreversible  
363 loss could be applied to the production of instant coffee. Furthermore, flavour houses  
364 that extract and stabilize coffee aromas could store aroma isolates and brew  
365 concentrates at high pH to preserve key aromas, and ready-to-drink coffees could apply  
366 these findings directly to their products to reduce the staling phenomenon.

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