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THE FORMS OF FLUORINE IN TEA AND ITS BIOACCESSIBILITY

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ABSTRACT: The tea plant is a hyper-accumulator of fluorine, especially in its mature leaves, which may negatively affect human health. In this study, the forms of fluorine in tea leaves and its bioaccessibility were analyzed. The results showed that tea polysaccharides accumulated about 68% fluorine of tea leaf. The bioaccessibility of fluorine in polysaccharides and tea water extract were lower than that of fluorine in sodium fluoride. The addition to polysaccharides by thearubigins and theabrownins, at a ratio of 1:2, reduced fluorine bioaccessibility. The addition of milk, more than 40 mL to 1 g polysaccharides, could also significantly reduce fluorine bioaccessibility, which may be attributed mainly to proteins and calcium compounds in milk. The present study suggests that the bioaccessibility of fluorine in tea is affected by the fluorine form and adding milk when drinking tea, which can effectively reduce side effects of fluorine in tea.

Keywords: Bioaccessibility; Fluorine; Milk; Tea chemicals; Tea polysaccharides.

Abbreviations: F: fluorine; TPS: tea polysaccharides; CTPS: crude tea polysaccharides; DTPS: dialyzed tea polysaccharides; DPTPS: deproteinized tea polysaccharides; WNZ: Wuniuzao; TWE: tea water extract; TR: thearubigin; TBs: theabrownins;

INTRODUCTION

Fluorine (F) is neither an essential trace element for humans nor necessary for the development of healthy teeth and bones.[1] An excessive intake will cause chronic fluoride poisoning,[2] such as dental fluorosis[3], skeletal fluorosis, and non-skeletal fluorosis.[4] The effect of F on human health is related to its form, free or non-free, with more toxicity caused by free F.[5] Tea plant (*Camellia sinensis* L.) is a hyper-accumulator of F, especially in the mature leaf which can accumulate F up to more than 1,000 mg/kg.[6] The form of F varies in tea. There are a water-soluble form, residual-F, organic matter-bound F, iron-manganese-bound, and exchangeable F.[7] Zhu and Gao reported that F in tea might exist in a form of compound with polysaccharide, with an F content of more than 1,000 mg/kg, which is about 80% of the total F content in the tea leaf.[8-9] However, the bioaccessiblity of F in tea is not clear.

Tea is the most popular beverage in the world, with a variety of nutrients and bioactive ingredients including tea polyphenols and its oxidation products, caffeine, amino acids, and sugars. These ingredients always play synergistic roles in bioactivity, such as detoxification and antioxidation. However, whether those ingredients also play an interference role in F bioaccessiblity still remains unclear. In addition, Europeans and residents in Sinkiang and Inner Mongolia of China have the habit of drinking tea with milk. An effect of milk on the bioaccessiblity of tea polyphenols has been reported.[10] Several studies have investigated the interaction between polyphenols and milk protein or mineral systems,[11,12] but no report is available, to the best of our knowledge, on whether milk affects the absorption of F.

Considering the potential interplay between tea chemicals with F, protein-F, mineral-F, and mineral-F-protein interactions, additional insight is needed for a better

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understanding of the potential role of dairy products in modulating the bioaccessiblity of F in tea. The purpose of this study was to investigate the form of F in tea, the bioavailability of F, and the impacts of the tea chemicals and milk on the bioaccessiblity of F.

MATERIALS AND METHODS

Materials and reagents: Tea preparation: Mature leaves (*C. sinensis*) of Wuniuzao (WNZ) were picked at the tea garden of Huazhong Agricultural University, followed by deactivating enzymes in a rolling machine at 200ºC, drying in a dryer at 80ºC, grinding, and sieving through a 0.42 mm sieve.

Fluorine standard solution was purchased from Shanghai Metrology and Testing Technology Research Institute; pepsin (P7125) and pancreatin (P7545) from Sigma-Aldrich.; xylanase (EC 3.2.1.8) from Shanghai Yuanye Biotechnology Co.; casein and whey protein from Sigma-Aldrich; theaflavin (≥50%) was from Jiujianghuahan Biotechnology Co.; tea polyphenols (≥90%) from Anhui Redstar Pharmaceutical Co., Ltd.; L-theanine (≥98%) from Shanghai Aladdin Bio-Chem Technology Co., Ltd.; fresh milk from Mengniu Co.; and Bradford kit from Nanjing Jiancheng Bioengineering Institute.

Preparation polysaccharides: Tea powder (100 g) was blended with 2 L water at 55ºC for 2.5 hr. After filtration, the liquor was concentrated to about 300 mL using a vacuum rotary evaporator (RE-52AA, Shanghai Yarong Biochemistry Instrument Factory, Shanghai, China) at 55ºC under reduced pressure, followed by the addition of a three-fold volume of 95% ethanol and incubation overnight. Next, the mixture was centrifuged at 4,000×g for 10 min, and the precipitate was freeze-dried (Coolsafe 110-4, Labogene Scan Vac, Lynge, Denmark) as the CTPS sample. The CTPS samples were dissolved in an appropriate amount of ultrapure water, dialyzed with 1000 Mr membrane for 72 hr and then freeze-dried as the DTPS. CTPS was deproteinized using the Sevag method.[13] Briefly, CTPS (1 g) was dissolved in 200 mL ultrapure water, followed by the addition of 40 mL chloroform and 8 mL n-butanol, shaking vigorously for 20 min, and centrifugation at 4000×g for 10 min. The water layer was treated 6 times with above procedure. Finally, the obtained deproteinized polysaccharide fluoride was dialyzed and freeze-dried as DPTPS sample.

Preparation of tea water extract and tea-pigments: Tea water extract (TWE): Tea power (5 g) was extracted with 250 mL water at 100ºC for 5 min. The mixture was then centrifuged at 4,000×g for 5 min. The supernatant was concentrated at 55ºC under reduced pressure and then freeze-dried.

Thearubigin (TR) was prepared as previously reported.[14-15] Briefly, 50 g black tea (Xuan'en Wujiatai Changcheng Tea Company) was blended with 500 mL distilled water at 90ºC for 10 min. The mixture was then centrifuged at 4,000×g for 5 min, followed by collecting the supernatant and extracting the residue once more under the same conditions. The two supernatants were pooled and concentrated to 200 mL at 55ºC under reduced pressure. The concentrate was extracted three times with 200 mL chloroform, ethyl acetate, and n-butyl alcohol separately. The n-butyl alcohol layer was dried and dissolved in 100 mL methanol. The solution was precipitated twice

with 300 mL diethyl ether each, the deposit was collected by centrifugation at 4,000×g for 15 min, and the residue pellet was freeze-dried to obtain the TR sample.

Theabrownins (TBs) were prepared as previously reported.[16] Briefly, 50 g dark tea (Hubei Zhaoliqiao Tea Industry Co. Ltd.) was supplemented with 500 mL water at 100ºC for 30 min. The mixture was then centrifuged at 4,000×g for 5 min, followed by collecting the supernatant and extracting the residue once more under the same conditions. The two supernatants were pooled and concentrated to 200 mL at 55ºC under reduced pressure. The concentrate was extracted three times with 200 mL chloroform, ethyl acetate and n-butyl alcohol separately. The aqueous phase was concentrated and supplemented with a four-fold volume of ethanol. After standing for 24 hr at room temperature, the mixture was then centrifuged at 4,000×g for 15 min. The sediment was collected and freeze-dried to obtain the TB sample.

Determination of bioaccessibility: Aliquots of samples were subjected to simulated gastric and small intestinal digestion in the sequence as previously described.[17] Briefly, the gastric phase solution (20 mL) (1 L solution contains, 1 g arabinogalactan, 3 g soluble starch, 2 g pectin, 3 g yeast powder, 1 g xylan, 1 g peptone, 0.4 g glucose, 4 g mucoprotein, 0.5 g L-cysteine, and 0.125 g pepsase, with pH adjusted to 3.0±0.1 by HCl) was added to the reaction conical flask with 0.2 g samples, vortexed, blanketed with nitrogen, and incubated in a covered shaking water bath (37ºC, 1 hr). Then, the intestinal phase solution (40 mL) (1 L intestinal juice contains 12.5 g $NaHCO_3$, 6.0 g bile, and 0.9 g pancreatin, with pH adjusted to 7.0±0.1 by $NaHCO_3$) was added to the above solution, vortexed, blanketed with nitrogen, and incubated in a covered shaking water bath (37ºC, 4 hr). After digestion, the mixture was centrifuged at 5,000×g for 15 min, and the supernatant was collected for measurement.

Bioaccessibility:

$$\text{Bioaccessibility} = \frac{mr}{mt} \times 100\%$$

Where: mr = the amount of fluoride released from the matrix to the solution
mt = the total amount of fluoride in the sample being tested

Determination of fluorine: Solid samples were pretreated as following. Briefly, 0.05 g of samples were added in a 50-mL nickel crucible, and wetted with a little ultrapure water, followed by the addition of 0.75 mL 16.75 mol/L NaOH, and incubation in an oven at 150ºC for 1 hr, 300ºC for 10 min, and 600ºC for 30 min. After cooling to room temperature, the ashed sample was neutralized using HCl and diluted to 100 mL with ultrapure water for measurement.

The analyte (10 mL) was mixed in a beaker with 10 mL TISAB buffer (3 mol/L sodium acetate and 0.75 mol/L sodium citrate for 1:1 mixed), and the F content of the samples was measured by F ion selective electrode.

Determination of other ingredients: Neutral sugar was determined by spectrophotometry with the color agent sulfuric acid-anthrone as previously described.[18] Uronic acid was determined by spectrophotometry with the color agent sulfate-carbazole as previously described.[19] Protein was determined by the Bradford method.

Statistical analysis: The data are presented as the mean±SEM of at least three repeated experiments. Significant differences were analyzed by one-way ANOVA, and LSD test was used for multiple comparisons (SPSS Statistics 17.0 software). Differences were considered to be significant when $p<0.05$.

RESULTS AND DISCUSSION

Fluorine content in tea components: Fluorine contents in tea leaf, tea water extraction (TWE), and crude tea polysaccharides (CTPS) were analyzed (Table 1).

Table 1. Fluorine content in different components of tea

Samples	Fluorine (g/kg)	Ratio of F in leaf (%)
Tea leaf	0.87±0.01	/
Water extract	2.72±0.05	78.0
Crude tea polysaccharides	21.30±0.29	68.5

The results showed that the F content varied significantly in tea leaf, TWE, and CTPS. The F content in TWE was two or three times that of the tea leaf, accounting for 78% of total F in tea leaf and that of CTPS was 20 times, accounting for 68.5% of the total F. In order to better understand the contribution of polysaccharides components to the accumulation of F, CTPS was purified using 1000 Mr dialysis membrane and Sevag deproteinization, and its composition was analyzed (Table 2).

Table 2. The analysis of polysaccharides composition

Samples	Protein (%)	Neutral sugar (%)	Uronic acid (%)	Fluorine (g/kg)
CTPS	2.78±0.12 Bb	16.17±0.14 Cc	8.99±0.24 Bb	21.30±0.29 Aa
DTPS	5.61±0.18 Aa	20.21±0.94 Bb	16.76±0.57 Cc	1.73±0.12 Bb
DPTPS	2.01±0.09 Cc	28.70±0.36 Aa	19.28±0.17 Aa	1.27±0.09 Bb

Note: Different letters in the same column mean a significant difference at $p<0.01$ (capital letters) and $p<0.05$ (small letters). CTPS: Crude tea polysaccharides; DTPS: Dialyzed tea polysaccharides; DPTPS: Deproteinized tea polysaccharides.

The contents of uronic acid and neutral sugar in dialyzed polysaccharides (DTPS) increased significantly while that of F decreased markedly, which was only one-twelfth that of the CTPS, indicating that most of the F was loosely bound with polysaccharides and dissociated during dialysis. The F content in DPTPS decreased moderately and the protein decreased significantly while that of uronic acid and

neutral sugar increased markedly, which meant that a part of F might be bound with the polysaccharides tightly and slightly affected by deproteinization.

The bioaccessibility of different fluorine forms in tea: The bioaccessiblity of F in sodium fluoride (NaF, CK) was significantly higher, 94.18%, than the bioaccessibility of F in TWE, 92.15% ($p<0.05$) (Figure 1). This implies that the forms of F in tea might be vary with the NaF, or the chemicals in tea infusion might interact with each other to modify the bioaccessiblity of F in tea extracts. According to Figure 1, the bioaccessiblity of F in TPS with a different purity was significantly lower than that of F in TWE. The higher the purity of polysaccharides was, the lower the bioaccessiblity was, with 85.22% in RTPS, 51.20% in DTPS, and 10.06% in DPTPS. The results mean that F in DTPS and DPTPS might combine with polysaccharides tightly and is more difficult to be released and absorbed by human body. TPS, a glycoconjugate, contains monosaccharide, uronic acid, proteins, inorganic elements, and so on.[20-21] Rich hydroxyls existing in monosaccharide and amino acids in protein can bind with F through hydrogen bonds or the metal ions in TPS can combine with F through ionic bonds. Our previous work[9] showed that polysaccharides have the characteristics of F adsorption which is related to its proteins and metal ions such as Mn^{2+}, Al^{3+}, and Fe^{3+}. Meanwhile, DPTPS is more compact with a smoother surface than CTPS,[9] which leads it being less water-soluble or acid-soluble and it also makes the combined F more difficult to be released.

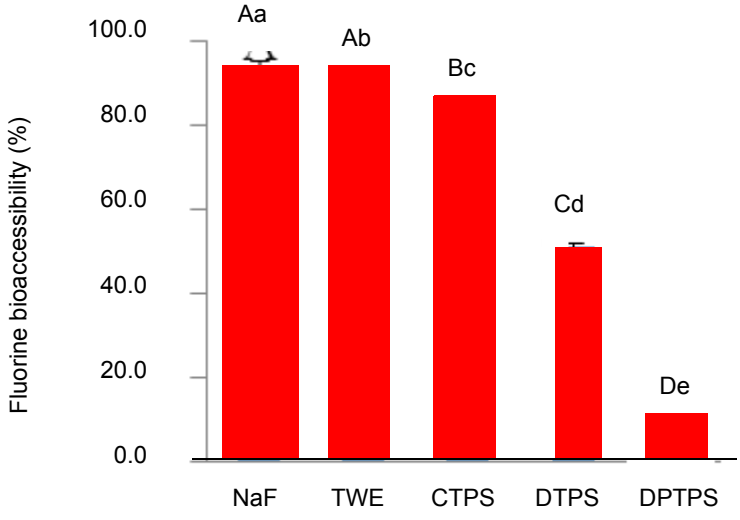

Figure 1. The bioaccessibility of fluorine in different substances.
Note: Different letters mean a significant difference at $p<0.01$ (capital letters) and $p<0.05$ (small letters). TWE: Tea water extract; CTPS: Crude tea polysaccharides; DTPS: Dialyzed tea polysaccharides; DPTPS: Deproteinized tea polysaccharides.

Effect of tea ingredients on bioaccessibility of fluorine in polyssacharides: Tea contains various active ingredients, including polyphenols and their oxides, caffeine, amino acids, sugars, and so on. Those ingredients, especially tea polyphenols and

their oxides, including theaflavins (TFs), thearubigins (TRs), and theabrownins (TBs), vary obviously in the composition of different teas. It is not clear whether those active ingredients influence the bioaccessiblity of F in tea drinking. The addition of TRs and TBs (2-fold TPS) resulted in the decrease of bioaccessiblity of F in TPS, but no obvious change occurred by the addition of tea polyphenols, caffeine, theanine, and TFs (Table 3). The more TRs and TBs were added, the lower the bioaccessiblity of F in TPS was.

Table 3. Effect of tea ingredients on bioaccessibility of fluorine in TPS

Added amount (g)	Added chemicals					
	Tea polyphenols	Caffeine	Theanine	TFs	TRs	TBs
0(CK)	85.08±0.79 Aa	85.24±0.69 Aa	84.70±0.50 Aa	85.05±0.37 Aa	85.12±0.05 Aa	84.27±0.94 Aa
1	84.88±0.70 Aa	86.01±0.30 Aa	84.72±0.10 Aa	84.97±0.85 Aa	85.22±0.21 Aa	85.64±0.41 Ab
2	84.21±0.14 Aa	85.58±0.12 Aa	84.97±0.47 Aa	85.23±0.29 Aa	84.31±0.13 Bb	80.85±0.33 Bc
3	84.68±0.16 Aa	85.56±0.60 Aa	85.37±0.44 Aa	85.16±0.11 Aa	82.60±0.20 Cc	79.06±0.18 Cd
5	84.40±0.2 Aa	85.45±0.50 Aa	85.02±0.60 Aa	85.67±0.27 Aa	79.82±0.25 Dd	69.41±1.13 De

Note: Different letters in the same column mean significant difference at $p<0.01$ (capital letters) and $p<0.05$ (small letters). TFs: Theaflavins; TRs: Thearubigins; TBs: Theabrownins.

TRs are a kind of complex polymers of theaflavins with a molecular weight about 700–40,000, and TBs result from the further oxidation polymerization of TRs. These macromolecules tend to complex with polysaccharides, protein, and metal ions to form polymers,[16] which may prevent the dissociation of F in TPS and thus lower the bioaccessiblity of F in TPS. Brick tea is a post-fermented tea. During the long period of 'post-fermentation', the catechins in tea could be oxidized and polymerized to generate theaflavins, thearubigins, and theabrownins, which might weaken the poisonous effect of F in tea liquid.

Effect of milk proteins and minerals on bioaccessibility of fluorine in TPS: Residents in Sinkiang and Inner Mongolia of China often drink tea with milk. In order to evaluate the impact of the milk matrix and concentration on the bioaccessiblity of F, a certain amount of milk was added. As shown in Figure 2, the addition of milk, at more than 40 mL to 1 g TPS, significantly lowered the

bioaccessiblity of F in TPS and had a dose-dependent effect. This result was consistent with the study of Ekstrand et al.,[22] who found that the milk can decrease F absorption rate under empty stomach conditions.

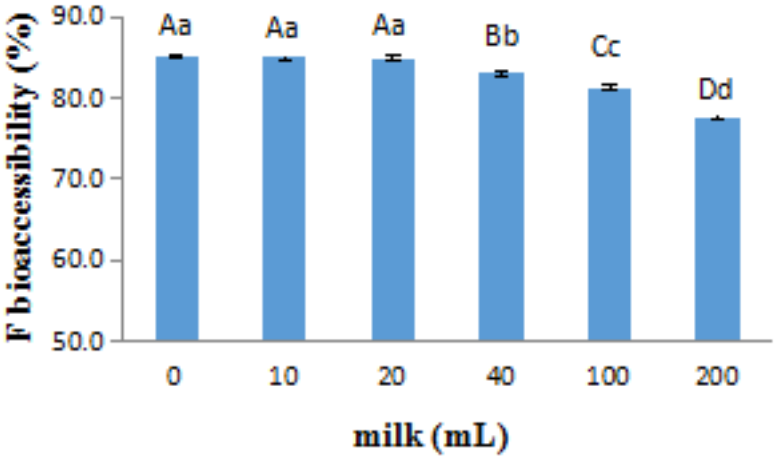

Figure 2. Effect of milk on the bioaccessibility of fluorine in polysaccharides.
Note: Different letters mean significant difference at p<0.01 (capital letters) and p<0.05 (small letters).

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The main proteins of milk were further analyzed and the impacts of casein and whey protein on the bioaccessiblity of F in TPS are shown in Figure 3. The addition of casein (1.0 g/g TPS) or whey protein (0.5 g/g TPS) significantly lowered the bioaccessiblity of F in TPS.

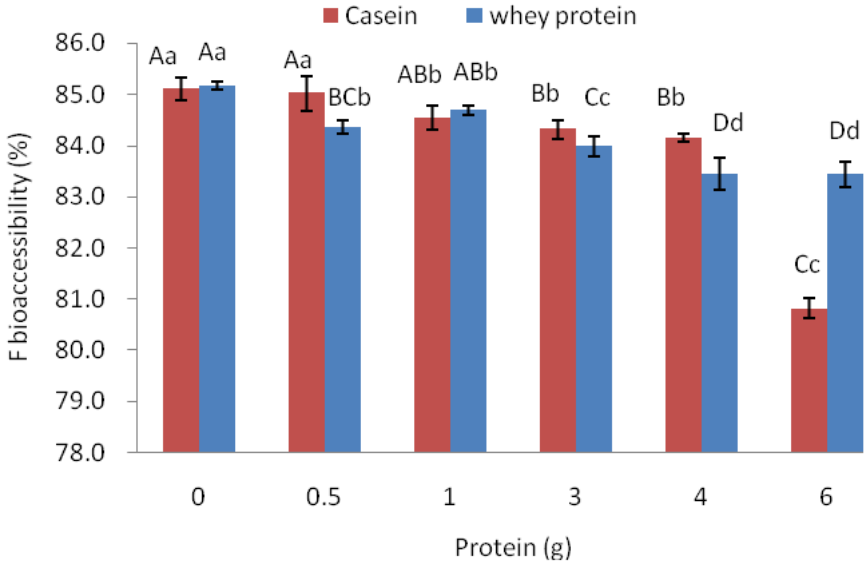

Figure 3. Effect of milk proteins on bioaccessibility of fluorine in polysaccharides.
Note: Different letters in the same color bar mean significant difference at p<0.01 (capital letters) and p<0.05 (small letters).

The addition of 100 mL milk, 2.27 g casein (equaling to the amount in 100 mL milk), and 0.34 g whey protein (equaling to the amount in 100 mL milk) decreased the bioaccessiblity of F in TPS by 4.6, 0.93, and 0.96%, respectively, indicating that both the casein and the whey proteins in milk have the same effect in reducing the

bioaccessiblity of F in TPS. This is probably because the protein contains many amino acids which can bind with F through hydrogen and make the F less absorbable.

Besides protein, milk also contains about 100 mg/100 mL of calcium, including calcium hydroxyphosphate (CHP), calcium citrate, calcium phosphate, and ionized calcium, which accounts for 40, 18, 3, and 9% of total milk calcium, respectively. All those calcium compounds had a significant lowering effect on the bioaccessiblity of F in TPS (Table 4).

Table 4. Effect of different forms of calcium on TPS fluorine bioaccessibility

Added amount (g)	Chloride calcium	Hydroxyapatite	Calciumphosphate	Calcium citrate
0 (CK)	84.86±0.33 Aa	84.55±0.24 Aa	84.87±0.14 Aa	84.75±0.25 Aa
20	82.87±0.67 Bb	84.20±0.46 Aa	84.78±0.25 Aa	83.39±0.20 Bb
40	74.45±0.40 Cc	82.44±0.25 Bb	82.5±0.25 Aa	80.33±0.26 Cc
60	51.66±1.17 Dd	81.15±0.24 Cc	83.29±0.34 Bb	-
100	41.06±0.23 Ee	75.47±0.3 De	78.25±0.06 Cc	-

Note: Different letters in the same column mean significant difference at $p<0.01$ (capital letters) and $p<0.05$ (small letters).

Of all the calcium compounds, CHP is the most abundant in milk and can significantly lower the bioaccessiblity of F in TPS at 40 mg/g TPS. The effective amounts of calcium citrate, calcium phosphate, and calcium chloride to significantly reduce the bioaccessiblity of F in TPS were 20, 60, and 20 mg/g TPS, respectively. The addition of 200 mL milk in 1 g TPS equaled to the addition of 80 mg CHP, 36 mg calcium citrate, 6 mg calcium phosphate, and 18 mg chloride calcium, indicating that CHP and calcium citrate are the most important forms of calcium in milk to reduce the bioaccessiblity of F, while chloride calcium and the calcium phosphate have little effect.

The decreasing effect of calcium on the bioaccessiblity of F might be related to the easy combination of calcium with F, and the conjugated calcium-fluoride is difficult to be absorbed by the body. A previous study showed that calcium can reduce the accumulation of F in bone and the urinary F excretion in rat,[23] which meant that an appropriate amount of calcium can inhibit the F accumulation in the animal body.[24-25] Gao et al. found that the addition of calcium in the feed affected the digestion and metabolism of F in sika deer.[26] Fluorine varies in its capacity to combine with different forms of calcium. CHP, an important component of human skeleton and tooth, is easy to combine with F to form a stable fluorapatite.[27-29] The research of Li

et al.[30] showed that CHP was more effective than calcium phosphate in reducing the F content in brick tea liquor. In this study, the effect of calcium compounds in milk on F bioaccessiblity varied, probably due to its different binding ability with F.

CONCLUSION

The forms of F in tea vary. Tea polysaccharides accumulated over 50% F in the tea leaf, and the bioaccessiblity of F in polysaccharides was lower than that of F in tea leaf extract and as F in sodium fluoride. A part of F tightly bound with polysaccharides is difficult to be utilized by human body. Tea polyphenols, caffeine, theanine, and theaflavins had less impact on the bioaccessiblity of F in TPF, but the oxidation polymers of tea polyphenols such as thearubigins, theabrownins could significantly reduce the F bioaccessiblity. Additionally, milk addition moderately decreased the bioaccessiblity of F and the proteins and calcium in milk played an important role in binding with F through hydrogen or ionic bonds. The results from this research indicate that the bioaccessiblity of F in tea are affected by the F forms and by whether or not milk is present.

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CONFLICT OF INTEREST STATEMENT

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, nor there is any professional or other personal interest of any nature or kind in any product, service, and company that could be construed as influencing the position presented in, or the review of, the manuscript entitled “The forms of fluorine in tea and tis bioaccessibility”.

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