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Research Paper

Cooking methods effectively alter perfluoroalkyl substances and nutrients in cultured and wild bullfrogs

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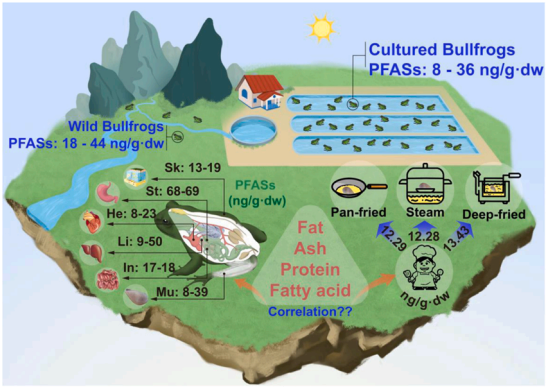
HIGHLIGHTS

- PFASs and nutrients were explored in raw vs. cooked of cultured vs. wild bullfrogs.
- Novel PFASs showed high detection ratio in wild bullfrogs compared with cultured.
- PFAS levels in wild bullfrogs were higher than in cultured for both raw and cooked.
- Changes in nutrients coupled with PFASs in cooked tissues were clearly identified.
- Steaming method for bullfrogs was recommended as a preferable and healthy treatment.

GRAPHICAL ABSTRACT



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ABSTRACT

The ubiquitous distribution of perfluoroalkyl substances (PFASs) poses a threat to the health of aquatic organisms and humans. Bullfrogs are considered a popular aquatic food product in South China, providing high protein and tasty cuisine; however bullfrogs have been shown to contain significant concentrations of PFASs. However, the risk-benefit ratios of PFASs and nutrient contents in cooked bullfrogs are not well understood. PFASs and nutrients were investigated in raw and cooked specimens of cultured and wild bullfrogs in this study. Novel PFASs showed higher detection levels and accumulation in wild bullfrogs than in cultured bullfrogs. Potential factors such as fat and fatty acid ratio affected PFASs accumulation in different tissues and by different cooking methods of bullfrogs. All cooking methods can reduce PFASs in edible tissues while significantly enhancing the nutritive value index (NVI) compared to raw bullfrogs. Steaming was the most effective way to reduce PFASs (rate of reduction was over 66%) and resulted in a lower risk of contributing to arteriosclerosis than other cooking methods assessed by atherogenicity index (AI) values. Cultured bullfrogs instead of wild bullfrogs were

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recommended for human consumption, and steaming was regarded as a better cooking method in terms of risk-benefit concerns. Overall, this work provides quantitative analysis of cooking methods that alter PFASs and nutrients in bullfrogs.

1. Introduction

Perfluoroalkyl substances (PFASs) have unique properties such as strong persistence and high surfactivity, resulting in wide use as refrigerants, surfactants, textiles, food packages, firefighting foams, and paints (Domingo and Nadal, 2017; Still et al., 2013). Some studies have indicated that long-chain PFASs can accumulate in the human body due to their long half-life period, and these compounds lead to toxic effects on the immune, nervous and endocrine systems (Guillette et al., 2020). Their persistence, bioaccumulation and toxicity are considered a global concern. Therefore, long-chain PFASs, such as perfluorooctane sulfonate (PFOS), perfluorooctanoate carboxylate (PFOA), and perfluorohexanesulfonic acid (PFHxS), are listed as persistent organic pollutants (POPs) (Stockholm Convention, 2017). However, PFASs are still used due to a lack of cost-effective alternatives. To replace long-chain PFASs, manufacturers have been using alternatives that include shorter-chain homologs. Factories are increasingly using <C8 PFASs and alternatives, such as perfluoroalkyl ether acids (PFEAs), instead of >C8 PFASs. However, some novel PFASs have shown potential risks to human health, including neurotoxicity, hepatotoxicity, thyroid function disruption, reproductive toxicity, and bioaccumulation (Han et al., 2021; Hong et al., 2020).

PFEAs, including both perfluoroalkyl ether carboxylic acids (PFECAs) and perfluoroalkyl ether sulfonic acids (PFESAs), have been detected in aquatic specimens such as bullfrog (*Rana catesbiana*), sucker (*Catostomus commersonii*) and silver carp (*Hypophthaimichthys molitrix*) (Liu et al., 2018; Munoz et al., 2017; Sun et al., 2021a). Various aquatic products are widely consumed due to their high levels of unsaturated fatty acids and proteins as well as some vitamins and essential minerals. Although humans can be exposed to PFASs by various pathways, dietary intake of aquatic products is regarded as the main pathway (Meng et al., 2019). Some studies have demonstrated that cooking reduces PFASs in aquatic products (Alves et al., 2017; Taylor et al., 2019). In contrast, a significant increase in PFOS levels was found after baking and frying fish (Bhavsar et al., 2014). The mechanisms and toxicological effects of altered PFASs after cooking processes remain unclear.

Bullfrogs are regarded as a type of indicator species for assessing ecosystem and human health risks because they live in a complex environment including both aquatic and terrestrial environments and bioaccumulate contaminants. Bullfrogs are chemically sensitive amphibians due to their low metabolic rate and highly permeable skin; thus, bullfrogs have enormous potential for the uptake and accumulation of pollutants (Bruhl et al., 2013; Cui et al., 2018). Bullfrogs are popular aquatic products due to their high nutritional value, sweet flavor and soft texture (Zhu et al., 2021). However, the potential risks of PFASs to human health due to the consumption of wild and cooked bullfrogs are still unclear. Wild bullfrogs feed on contaminated food from complex environments; thus, they may accumulate more pollutants than cultured bullfrogs. Frying and steaming are traditional cooking methods for bullfrogs; in particular, China consumes a large amount of cooked bullfrogs. Sobral et al. (2018) reported that different cooking methods affect lipid oxidation, protein structure damage and fatty acids loss. Altered nutrients could influence the accumulation of contaminants in cooked food. However, there is no report on the mechanism of altered PFASs in cooked bullfrogs, or PFASs levels related to human health. Therefore, further investigation is necessary for a better understanding of the mechanism of PFASs loss during cooking processes, including steaming, pan-frying, and deep-frying.

In this study, we investigated the concentrations of legacy and novel PFASs and nutritional ingredients in raw vs. cooked and cultured vs. wild bullfrogs (*Rana catesbiana*). The objectives were to (1) compare the bioaccumulation of individual PFASs among different tissues of cultured and wild bullfrogs; (2) assess changes in the concentrations and distributions of target PFASs in bullfrogs after being cooked; (3) determine the relationships between PFASs and nutrients in tissues impacted by different cooking methods; and finally, (4) refine the possible risk-benefit ratio of bullfrog consumption based on PFASs and nutrient levels. This study presents the first comparison of novel PFASs and nutrients between cultured and wild bullfrogs associated with different cooking methods, which will provide baseline information for risk assessment of bullfrog consumption on human health and the development of guidelines for food security management.

2. Methods and materials

2.1. Sampling strategies

Bullfrog farming is rapidly expanding in China with the improvement of techniques in bullfrog cultivation. In South China, with a warm climate and abundant water resources, it is suitable for the growth of bullfrogs so the breeding scale is gradually expanding. The production of bullfrogs in China was 60,000 tons in 2021, and the annual output of bullfrogs in Guangdong Province located in South China has been ranked at the top in the country, occupying approximately one-third of the country's output (Wang et al., 2022a, 2022b). Guangdong, as an intensive developing area, is highly industrialized and has substantial human disturbance, which leads to high levels of accumulation of pollutants in bullfrogs. All samples consisting of cultured (n = 80) and wild (n = 53) bullfrogs, with lengths and weights of 10.8–12.0 cm and 170.7–182.1 g, respectively, were collected from typical bullfrog farms and adjacent fields in Guangdong Province of South China, as shown in Fig. S1, in June 2021. All containers and tools were cleaned by rinsing sequentially with methanol and Milli-Q water before use. The skin, intestines, stomach, liver, muscle and heart were carefully dissected and separated individually from healthy bullfrogs. All samples were stored in sealed polypropylene (PP) bags and frozen at − 20 °C until treatment. Some samples were pretreated directly, while some mixed edible parts, including muscle and skin, were cooked and then pretreated. All samples, including raw and cooked samples, were analyzed in the same way to ensure homogeneity for analysis.

2.2. Materials and reagents

All 21 mass-labeled internal standards and 9 native standards were purchased from Wellington Laboratories (Guelph, Ontario, Canada), including 2,2,3,3-tetrafluoro-3-(trifluoromethoxy) propionic acid (PFMOPrA), perfluoro (4-methoxybutanoic) acid (PFMOBA), sodium dodecafluoro-3 H-4,8-dioxanonanoate (ADONA), chlorinated polyfluorinated ether sulfonate (F-53B), perfluoro-butanesulfonate (PFBS), PFOS, perfluorobutyric acid (PFBA), PFHxS, perfluorodecane sulfonate (PFDS), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA), perfluorohexadecanoic acid (PFHxDA), and perfluorooctadecanoic acid (PFODA). Chromatographic grade methanol and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ, USA), and petroleum ether, n-hexane, and ammonium acetate were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Mixed fatty acid standards were purchased from

Shanghai Yuanye Bio-Technology Co., Ltd. (Qingpu District, SH, China). Detailed information about the standards is shown in Table S1.

2.3. Chemical extraction and cleanup

Some bullfrogs were dissected and the heart, skin, liver, muscle, stomach, and intestines were harvested. Other whole-body bullfrogs were pretreated directly. To reduce cross-contamination throughout the experiment, PP centrifuge tubes were washed with Milli-Q water and methanol more than three times before chemical analysis. The procedure for PFASs analysis in bullfrogs was based on previous studies (Sun et al., 2021b; Diao et al., 2022). In brief, one gram of the sample spiked with 5 ng of internal standards was added to a 50 mL PP centrifuge tube for sufficient mixing and extraction. The samples were further analyzed using a solid phase extraction method by ENVI-Carb and Oasis WAX to reduce interference. Details of the extraction processes are given in the Supplementary Information.

2.4. Analysis of proximate compositions and fatty acids

Nutrients were determined based on previous methods (Islam et al., 2021). Ash was analyzed by incineration in a muffle furnace at 600 °C for 24 h (CWF1100; Carbolite). Crude protein was estimated as Kjeldahl nitrogen using a factor of 6.25 (Kjeltec™8400; FOSS), and crude lipid was measured by the Soxhlet method using petroleum ether at 40–60 ℃ for 4 h (SZF-06A; Xinjia Yiqi). Fatty acids were extracted using chloroform/methanol (2/1, v/v) according to previous methods (Dong et al., 2018; Zula et al., 2021). A total of 0.5 g of the sample and 6 mL of chloroform/methanol were added to a 10 mL glass tube and stored at 4 ℃ for two days. Then, 2.7 mL of ultrapure water was added to the glass tube with shaking (250 rpm) and centrifugation (3000 rpm), and the underlying fluid was drained by a Pasteur pipette. The extraction was extracted with 3 mL of chloroform twice as described above. After extraction, the glass tube was heated at 85 ℃ for 3 h in a warm water bath with 2.5 mL of 2% vitriol/methanol. After incubation, the mixture was cooled to room temperature. One milliliter of saturated sodium chloride and 2 mL of n-hexane were added to the tube. Liquids were concentrated to 0.5 mL under high-purity nitrogen airflow, passed through a nylon filter and then transferred into a 1.5 mL brown glass vial via a sterile syringe.

2.5. Treatment of different cooking methods

Healthy bullfrogs were carefully processed to harvest the muscle, skin, intestines, stomach, liver and heart, but only the edible parts, including skin and muscle, were cooked by different methods. All edible parts were mixed (approximately 2 kg) to reduce individual differences and then divided into three groups of parallel samples in each cooking method. There was approximately 300 g of edible bullfrog in each group. Steaming is a common cooking method by which foods are heated by vapor from boiling water and can conserve more moisture in the food than other methods. In this study, 100 g of bullfrog was steamed in a container with 300 g of water for 10 min on medium heat and for an additional 5 min on low heat. There are various types of frying techniques, such as deep-frying and pan-frying, that can change the physical and chemical properties of foods and release a distinct aroma. Deep-frying and pan-frying methods, both widely used, have different levels of contact with the cooking oil. Foods were submerged into a sufficient amount of oil during the deep-frying process, while only a small amount of oil was added in the pan to cook foods during the pan-frying process. In this study, 250 g of oil was prepared in each deep-frying process and 100 g of bullfrog was cooked for 5 min at 150 °C and 170 °C. One hundred grams of bullfrog and 10 g of oil were used during the panfrying process, which resulted in a cooking time of approximately 8 min. All experimental conditions of this study were based on the investigation of the dietary habits of people in China and contamination of samples with external PFASs was controlled by additional measures. For example, non-PTFE (polytetrafluoroethylene) materials and stainless steel equipment were used, and all utensils were repeatedly rinsed with Milli-Q water and methanol to avoid contamination. Moisture contents of the bullfrog are shown in Fig. S2.

2.6. Instrumental analysis and quality assurance

Chemical analyses of PFASs were performed using a Thermo Ultimate 3000 Infinity HPLC System coupled to a Thermo TSQ ENDURA LC/MS System. Quantitation was accomplished using multiple reaction monitoring with electrospray ionization in negative ion mode. An Agilent ZORBAX Eclipse Plus C18 (2.1 ×100 mm, 3.5 μm) connected to a guard column was used. To separate contaminants, 2 mM ammonium acetate (A) and 100% acetonitrile (B) were used as gradient elution of the mobile phase. A 5 μL aliquot of extracts was injected into the column at a flow rate of 0.3 mL/min. Information, such as parent ion, chemical formula, qualitative ion, and quantitative ion, are presented in Table S1. Instrument conditions of HPLC-MS/MS are shown in Table S2.

Fatty acid methyl ester was detected by a Shimadzu GCMS-QP2010SE equipped with an HP-MS capillary column (29 m × 0.25 mm×0.25 μm), which was confirmed by retention time and mass spectra standard substance matching. The column temperature was 100 °C, and the MS was operated in electron impact ionization (EI) mode. Further details on instrumental conditions are presented in Table S3.

Experimental design and methods reported by previous research were used to increase reproducibility and comparability (Cowger et al., 2020). To obtain reliable data, process blanks, solvent blanks, and duplicate samples were analyzed. Procedural blanks were used for each batch of 12 samples, and the procedural blank concentrations for the samples are presented in Table S4. PP bags of the blank control were assessed in this study, whose individual PFASs concentrations were all lower than the limit of detection (LOD). Quality assurance and quality control (QA/QC) for PFASs are shown in Table S4.

2.7. Risk calculation and data analysis

Each sample was processed three times by LC/MS System, and average values of the data were analyzed by various software. All data manipulation and statistical analysis were performed using OriginPro 9.0 (OriginLab Corporation, USA), SPSS (SPSS Inc. Quarry Bay, HK), and Excel 2016 (Microsoft Corporation, USA). ArcGIS V10.2 software (ESRI, Redland, CA, USA) was used to analyze the mapping of the sampling locations. The normality and homoscedasticity of the data were tested using the Shapiro-Wilk test and Levene's test. The criterion of homogeneity of variances was generally not met in the datasets; thus, nonparametric statistical analyses were performed in this research. Differences between cultured and wild bullfrogs were assessed by the Mann-Whitney U test with a statistical significance threshold of $p < 0.05$. Relationships between PFASs and nutrients were evaluated by Spearman rank correlation coefficients (R_s). R_s explained the proportion of the rank variance in the correlation between variables with test assumptions more suitable for this dataset. Linear regression was used to evaluate the relationships between PFASs and nutrients.

The atherogenicity index (AI) has a positive correlation with the risk of cardiovascular disorder calculated by the ratio of saturated fatty acids and unsaturated fatty acids because saturated fatty acids are proatherogenic and unsaturated fatty acids are antiatherogenic (Zula et al., 2021; Bland et al., 2021; Ulbricht and Southgate, 1991). The equation is as follows:

$$\mathrm{AI} = \frac{\mathrm{C12:0} + (\mathrm{C14:0} \times 4) + \mathrm{C16:0}}{\Sigma\mathrm{MUFA} + \Sigma\mathrm{PUFA}\,(\mathrm{n-3}) + \Sigma\mathrm{PUFA}\,(\mathrm{n-6})} \quad (1)$$

The nutritive value index (NVI) evaluates the nutrition of fatty acids (Abdel-Naeem et al., 2021; Werenska et al., 2021). The equation is as follows:

$$NVI = \frac{C18:0 + C18:1}{C16:0} \quad (2)$$

The thrombogenicity index (TI) indicates the trend of forming clots in blood vessels (Du et al., 2012). The equation is as follows:

$$TI = \frac{C14:0 + C16:0 + C18:0}{0.5 * \Sigma MUFA + 0.5 * PUFA(n-6) + 3 * PUFA(n-3) + PUFA(n-3)/PUFA(n-6)}. \quad (3)$$

3. Results and discussion

3.1. Occurrences of PFASs in cultured and wild bullfrogs

The internal distribution of PFASs is shown as a heatmap of tissue/muscle ratios (TMRs) in Fig. 1 to assess the contribution of individual tissues to muscle. The distribution pattern in PFASs composition revealed tissue dependence. Muscle was selected as a reference value because it is usually used as the edible part of bullfrogs. Compared to the actual concentration, TMRs allow us to more quickly screen for less polluted parts than muscle as healthy food. Of note, the highest TMR values in similar carbon chains, such as PFMOPrA vs. PFBA, PFMOBA vs. PFPeA, and F-53B vs. PFOS, had similar patterns of tissue distribution in bullfrogs. The TMR values of PFMOPrA and PFBA were highest in the stomach of wild bullfrogs. PFMOBA and PFPeA in the liver of wild bullfrogs, which are five-carbon chains, showed the highest TMR values. Furthermore, F-53B, as a PFOS alternative, had a similar trend: the highest TMR value was shown in the liver of wild bullfrogs as PFOS, suggesting that these similar carbon-chain substances have common mechanisms of distribution and bioaccumulation.

High TMR values in the stomach and liver of bullfrogs represented higher PFASs concentrations and risk in these tissues than in other parts. Concentrations of PFASs in samples greatly varied across target tissues, with the highest concentrations in the digestive system (e.g., intestines and stomach) of bullfrogs. Owing to deep roots in human cultures, the consumption of aquatic products, such as bullfrogs, has been impossible to reverse in recent years. The interest of public health in decreasing PFAS levels in bullfrogs should be considered; for example, the stomach of dissected bullfrogs must be removed and rinsed with clean water.

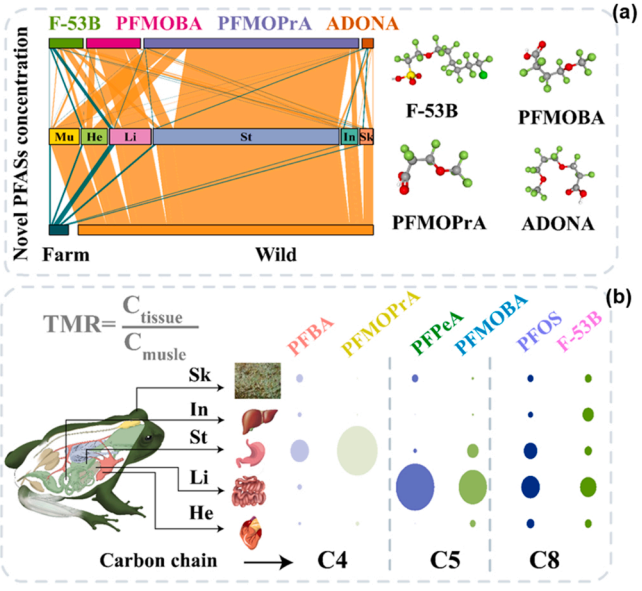

Fig. 1. PFASs and TMR values in bullfrog tissues from South China **Note:** (a) Novel PFASs concentration in bullfrog; (b) TMR is the ratio of concentrations in edible muscle parts and other individual tissues. Sk, In, St, Li, and He represent the skin, intestines, stomach, liver, and heart, respectively.

PFBA was the most abundant chemical among the PFASs measured in the target tissues of wild bullfrogs, except for the heart. The highest concentration of PFBA was detected in the stomach (40.62 ng/g·dw), followed by the muscle (32.77 ng/g·dw) (Fig. S3). High detection rates of individual PFASs were found in the skin of wild bullfrogs (Figs. S4 and S5). The underlying reason may be that gas and other small molecules can permeate the skin of bullfrogs by cutaneous respiration, which is a particularly common exposure pathway for pollutants such as PFASs.

The concentration of classic $\geq$ C8 PFASs in wild bullfrogs was significantly higher than that in cultured bullfrogs (Mann-Whitney U test; $p < 0.05$) (Fig. S6); for example, PFOS concentration in whole wild bullfrogs (6.85 ng/g·dw) was higher than that in whole cultured bullfrogs (3.13 ng/g·dw), as shown in Fig. S7. Novel PFASs had similar results: higher levels were detected in wild bullfrogs than in cultured bullfrogs ($p < 0.05$), which showed similarity with a previous report on wild and cultured fish (Du et al., 2012). Wild bullfrogs live in complex wild environments and consume a variety of contaminated food, whereas cultured bullfrogs are fed less contaminated food on bullfrog farms where water and sediment are changed frequently. Organisms can be exposed to PFASs within water and sediment via the respiratory tract. Additionally, organisms can be exposed to PFASs by ingestion, which then transfers into the blood stream and finally reaches individual tissues (Robuck et al., 2021). These observations collectively explain the higher concentrations of PFASs in the wild than in the cultured bullfrogs.

The detection rate of novel PFASs was high in wild bullfrogs, reaching 100%. Concentrations of short carbon-chain PFASs were higher than those of long carbon-chain PFASs in bullfrogs ($p < 0.05$) (Fig. S8). This result may be caused by related factories and nearby enterprises that started to use and discharge short carbon-chain PFASs, followed by the potential effects of some novel PFASs. Accordingly, novel PFASs are increasingly abundant in the surrounding environment and even accumulate in bullfrogs. Indeed, dietary intake is an important pathway of humans in daily life. Wild bullfrogs are contaminated with PFASs that pose risks to humans by impacting the immune function, metabolic outcomes, and neurodevelopment (Skogheim et al., 2020; Zeng et al., 2019), which increase potential risks for people. The above results strongly suggest that people who prefer wild food for nutritional value and delicious taste should pay attention to food safety and potential health issues.

3.2. Changes in PFASs after using different cooking methods

The effects of cooking methods on the concentration and composition of PFASs in bullfrogs were investigated (Fig. 2). Concentrations of PFASs in cooked bullfrogs varied greatly by cooking method. The most effective cooking method to reduce PFASs was found to be steaming, by which the PFASs concentrations in raw samples (36.22 ng/g·dw) was reduced to 12.28 ng/g·dw in cooked samples (Fig. S9). Comparing different cooking methods, the PFOS concentration was significantly lower in cooked bullfrogs after steaming than after using other methods (Figs. S10 and S11). The results of this study are in agreement with the previous observation that PFASs in aquatic products decreased after

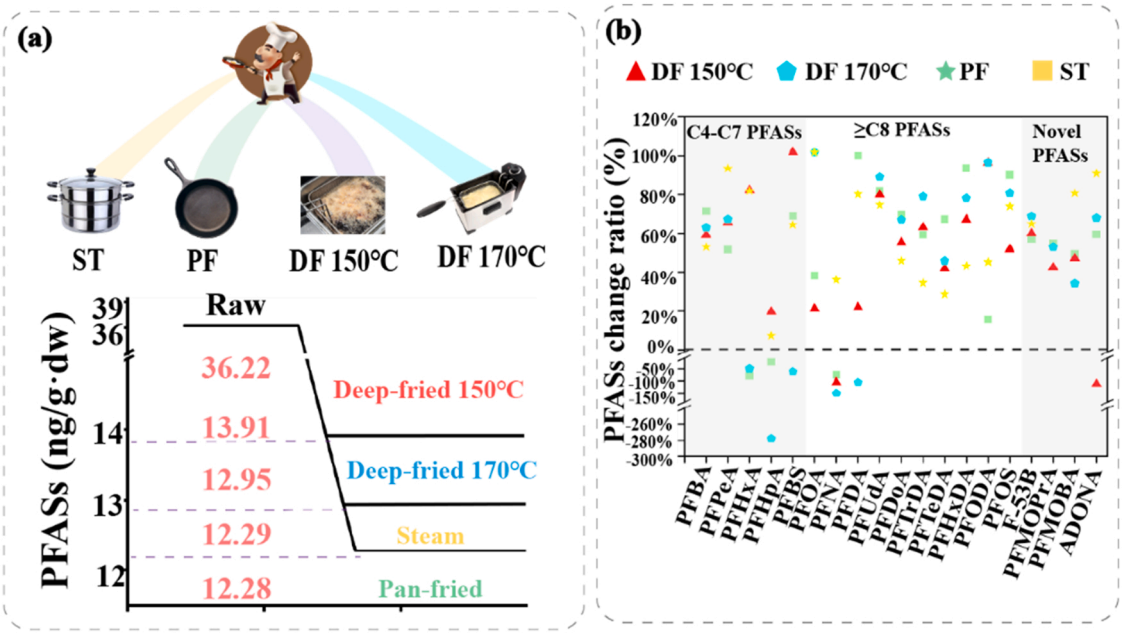

Fig. 2. Individual PFASs in bullfrogs by different cooking processes **Note:** (a) Total PFASs concentration change by different cooking processes. ST, PF, DF represent steaming, pan-fried, deep-fried, respectively; (b) Individual PFASs change ratio.

cooking (Alves et al., 2017). However, another study has provided the opposite conclusion: the PFASs concentration significantly increased after cooking (Bhavsar et al., 2014).

After 15 min of steaming, over 66% of ∑PFASs were reduced in bullfrogs (Fig. 2). There is also a high total PFASs loss ratio after steaming, which is a common cooking method for bullfrogs. Possible PFASs loss mechanisms during steaming include vapor permeation through samples that reflow into the water with contaminants. The increased temperature during the cooking process can improve the solubility of contaminants and accelerate evaporation. Hence, water-soluble substances can be eluted from bullfrogs into the water (Luo et al., 2019). PFASs are highly accumulated in bullfrog tissues due to their strong biological affinity for proteins. The increasing temperature during cooking processes may affect tissue protein structures and denature them. Protein-PFAS bonds within tissues of bullfrog were likely disturbed, and PFASs were discharged from bullfrogs into the external media at a high temperature, which caused a high PFASs loss ratio in bullfrogs during cooking processes (Yang et al., 2020). Therefore, some PFASs concentrations decreased in the tissues of bullfrogs after cooking even though PFASs exhibit chemical stability and heat resistance. When comparing steaming to other cooking methods, it is determined that longer steaming time likely decreases PFASs concentration. Vendl et al. (2022) found by a meta-analysis that cooking time and PFASs concentration had a strong correlation in cooked animal tissues.

Similar to the results in raw bullfrogs, PFBA, a type of short-chain PFASs, was the predominant PFASs in the cooked samples. PFPeA, another short-chain compound, was the next most prominent PFASs detected in most cooked samples. In contrast to previous studies, PFOS was reported to show the highest PFASs concentration in aquatic products after the cooking process (Taylor et al., 2019). Differences were attributed to a complex system of different contaminant levels in raw biota samples, species of organisms, and cooking methods. PFTE-coated cookware was also a vital factor in contaminant levels when PFASs were transferred from PTFE-coated cookware at high temperatures during cooking processes (Ruffle et al., 2020). To determine how PFASs changed during cooking, in this study, all cookware was strictly controlled to exclude PTFE coatings.

F-53B, PFMOPrA, PFMOBA, and ADONA, as novel PFASs, were examined in all bullfrogs cooked by different methods (Fig. S12). The concentrations of these novel PFASs decreased after the steaming process. Notably, even if cooking processes can reduce the amount of novel PFASs, the detection rates of novel PFASs reached 100% in the cooked bullfrogs. PFASs, as persistent organic pollutants, have a long half-life and degradation-resistant properties; thus, PFASs can accumulate in human bodies over time. Aquatic products play an important role in meeting the daily nutritional needs of many people; however, we should continue to pay attention to food safety and choose pollutant-free sources or pollutant-reducing cooking methods such as steaming.

3.3. *Nutrients coupled with PFASs in bullfrogs*

Relevant studies reporting a possible relationship between PFASs contaminants and nutrients in bullfrogs are lacking. Hence, the changes in PFASs and nutrients in different bullfrog tissues in this study are highlighted in Fig. 3. There was a statistically positive correlation between fat levels and PFASs concentrations in bullfrogs ($R = 0.68$, $p < 0.05$). (Table S5). Furthermore, linear regression showed that the fat content of bullfrogs significantly affected PFASs concentrations ($p < 0.05$), and fat content accounted for 59% of the variation in PFASs concentrations ($R^2 = 0.59$, Fig. S13). The distribution of ≥C8 PFASs levels in tissues showed a similar trend to that of fat, of which a high concentration was found in the liver, and a low concentration was found in the muscle (Fig. S14). The level of fat may affect the accumulation of individual contaminants. The findings in this study coincide with the positive relationship between PFHxA and PFPeA found in top mouth culter, PFHxS levels in bighead carp, and PFDA levels in human serum (Bijland et al., 2011; Fu et al., 2014; Wu et al., 2019). Moreover, it was reported that high POP levels were due to more fat in aquatic products (Menezes-Sousa et al., 2021). In this study, there was no significant correlation between novel pollutants and proteins, as shown in Table S5 ($p > 0.05$), but it has been reported that novel PFASs, such as ADONA and F-53B, bind to proteins by an efficient molecular dynamic approach (Cheng et al., 2018). Differences in electrostatic surface potential among compounds may affect their protein binding capacities, which explains why individual PFASs were diversified in different bullfrog tissues. Robuck et al. (2021), using URBO-MOLE software, found that Nafion byproduct-2 (Nafion BP2) had a higher level of positive surface charge density and a lower level of neutral surface charge density than PFOS

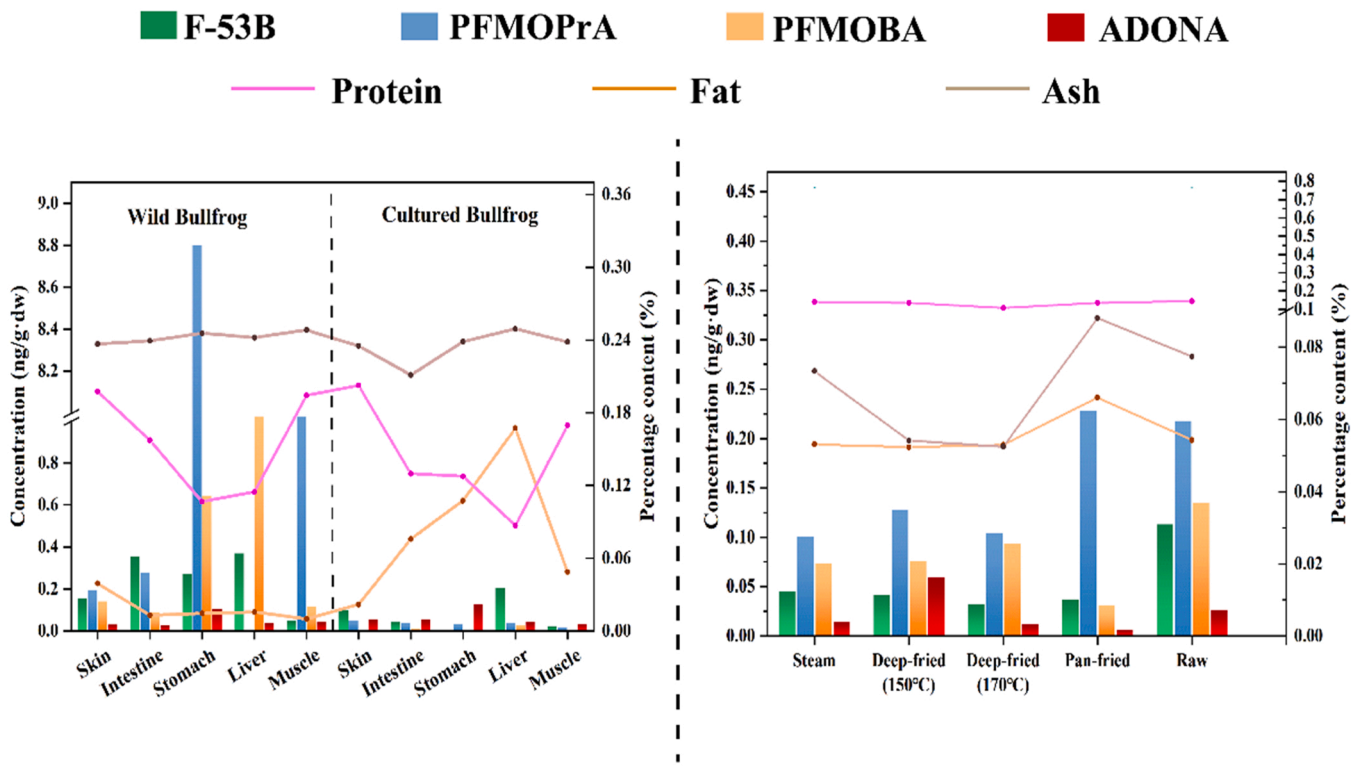

Fig. 3. Correlation between PFASs and nutrient contents in bullfrogs **Note:** (a) Correlation between PFASs and nutrient contents in bullfrog tissues; (b) correlation between PFASs and nutrient contents by different cooking processes. ST, PF, and DF represent steaming, pan-fried, and deep-fried, respectively.

(Robuck et al., 2021). Consequently, hydrophobic interactions of Nafion BP2 reduced the active site for protein binding, and it was found that Nafion BP2 in juvenile seabirds was reduced in the liver compared to blood.

Cultured bullfrogs had higher fat levels than wild bullfrogs ($p < 0.05$), as shown in Fig. 3. There were similar results in a previous study showing that most cultured aquatic products may have more fat (even twice as much) than wild samples among the same species (Moradi et al., 2011). Potential reasons are that cultured bullfrogs are fed processed baits to maximize growth rates, and wild bullfrogs usually live in the vast wilderness and run, thus more fat is burned up. The fat content of wild bullfrogs was high in the liver but low in the skin. In this study, bullfrog samples contained a high protein content that was adequate for human health to a certain extent. Proteins are beneficial for the human body and can decrease obesity and tumor necrosis factors, reduce the oxidative stress of adipose tissue, control type 2 diabetes, improve the resolution of inflammation, and lower cardiovascular risk (Islam et al., 2021; Khalili Tilami and Sampels, 2017). The protein content was similar in the skin of cultured and wild bullfrogs, but it was higher in the muscle of wild bullfrogs than cultured bullfrogs. Fat and protein contents of bullfrogs are diverse even within species due to various abiotic and biotic elements such as the type and amount of diet, salinity, pH of water, temperature, and reproductive cycle.

Cooking involving a temperature change easily affects the nutrient content and physicochemical properties, such as ash, fat and protein, which will ultimately affect the distribution of pollutants in a simultaneous manner (Fig. 3). The relative content of ash in the deep-frying process was less than that in the pan-fried process. Although both samples were fried, they had different contact areas with oil. The fryer has a sufficient amount of hot oil to fully submerge foods during the deep-frying process, while the pan has only a small amount of oil to cook foods during the pan-fried process. In this study, the ash content was at the lowest level in deep-fried bullfrogs because high temperature considerably damaged bullfrogs, and most minerals transferred into the oil.

In the deep-frying method, temperature is essential, and protein content decreases because of protein destruction at high temperatures. Myofibrillar and connective tissue proteins can be damaged and result in fiber shrinkage and aggregation of sarcoplasmic proteins, which explains why cooked bullfrogs are easier to digest and chew and have higher nutritional value than raw bullfrogs (Sobral et al., 2018). Variations of fat were more obvious than those of proteins by different cooking methods, especially in pan-fried processes. The potential reason why pan-fried bullfrog samples had higher fat content than raw bullfrogs was that fat was absorbed into bullfrogs from oil. This result was consistent with other studies showing that nutrient composition is affected by cooking methods because fried food items are considerably damaged, resulting in more fat uptake from oil (Liu et al., 2021).

3.4. Correlation between fatty acids and PFASs in bullfrogs

3.4.1. Patterns in different tissues

Correlations between PFASs and fatty acids in wild and cultured bullfrogs are shown in Fig. 5. Comparing different fatty acids, concentrations of polyunsaturated fatty acids (PUFAs) were enriched in different tissues of wild and cultured bullfrogs. There was also a statistically significant correlation between saturated fatty acids (SFAs) and C4-C7 PFASs in bullfrogs by Spearman bivariate correlation analysis ($R = 0.64$, $p < 0.05$) (Table S5). Data adjusted to a linear model between SFA and C4-C7 PFASs show positive slopes and an R^2 of 0.29 in Fig. S15. Of note, SFAs were at a high level in the liver and at a low level in the stomach of cultured bullfrogs. In cultured bullfrogs, C4-C7 PFASs also had an opposite trend and were at a low level in the liver and at a high level in the stomach (Fig. 4). There were similar results in previous studies as follows. High PUFA (n-3) content and low levels of PFASs were detected in marine fishes, such as *Xiphias gladius, Scomber scombrus, Reinhardtius hippoglossoides, Engraulis encrasicolus,* and *Salmo salar*, whereas high levels of PFASs and low levels of PUFA (n-3) were found in

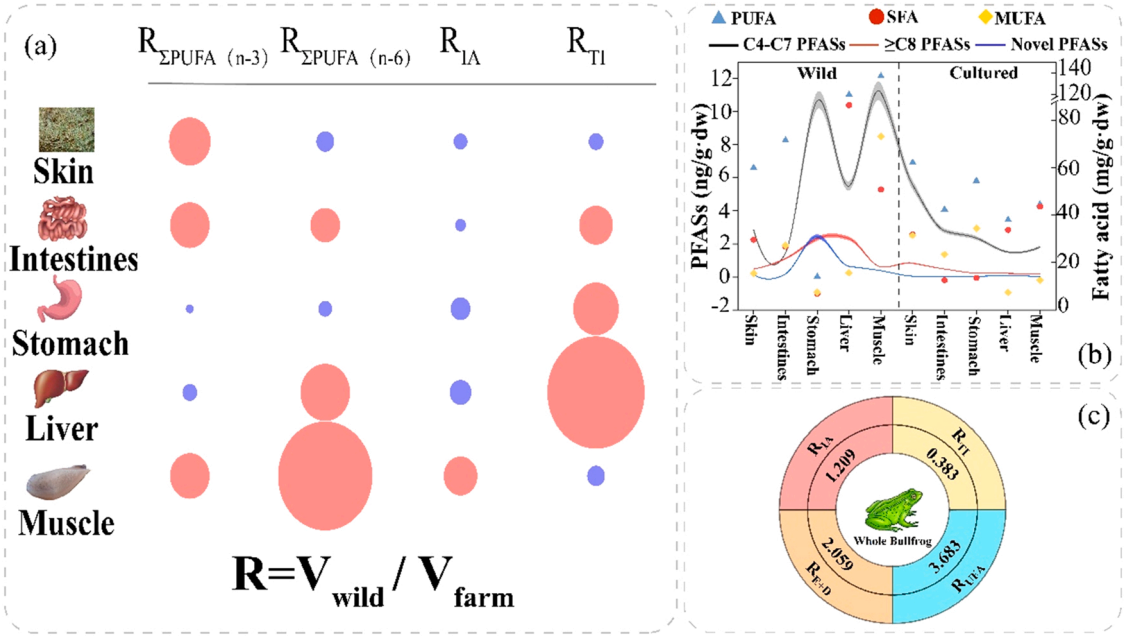

Fig. 4. Correlation between fatty acids and PFASs in bullfrog tissues **Note:** (a) The ratio of values in tissues of wild and cultured bullfrogs; the red and blue circles represent values greater than 1 and less than 1, respectively; (b) correlation between PFASs and fatty acids in bullfrogs; (c) the ratio of values in wild and cultured whole bullfrogs.

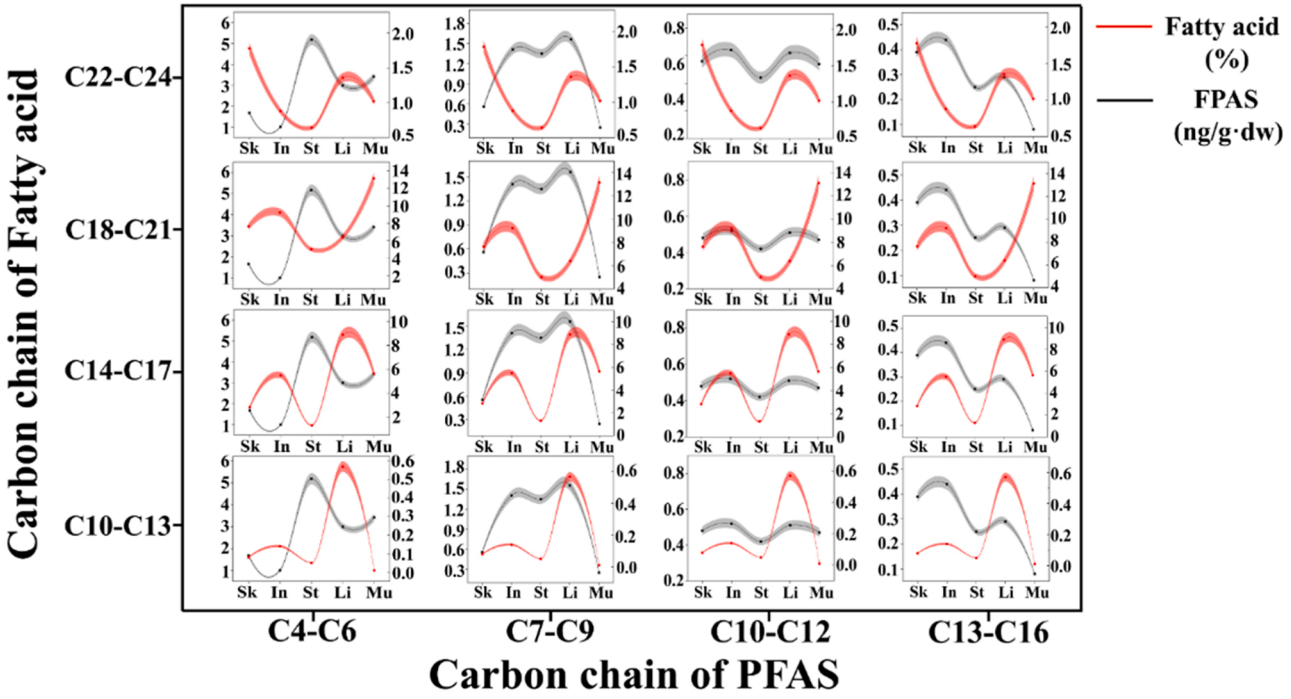

Fig. 5. Correlation between different carbon-chain fatty acids and PFASs in bullfrog tissues.

freshwater fishes such as *Cyprinus carpio, Sander lucioperca, Perca fluviatilis, Chelon labrosus,* and *Rutilus rutilus* (Yamada et al., 2014). PFASs were reported to bind fatty acid binding protein (FABP) and compete with fatty acids because PFASs have a highly hydrophobic tail and a hydrophilic head targeting FABP (Cheng and Ng, 2018). PFOS has a stronger protein binding capacity than PFOA because the sulfonic group of PFOS has one more oxygen to form hydrogen bonds than the carboxyl group of PFOA. These results can explain why the level of PFOS in the liver of wild bullfrogs is significantly higher than that of PFOA ($p < 0.05$) (Fig. S16).

The muscle of wild bullfrogs has a significantly higher PUFA concentration than that of cultured bullfrogs (Fig. 4). It has been reported that a high PUFA concentration may increase food deliciousness and juiciness in wild aquatic products, which can account for the dietary preferences of people to consume the muscle of wild bullfrogs (Wang et al., 2014). In muscles, SFA concentration in wild bullfrogs was clearly higher than that in cultured bullfrogs, which may lead to arterial lumen stenosis and atherosclerosis, increasing the risk of coronary heart disease. This occurs because SFA can lead to arteriosclerosis by increasing the relative content of cholesterol and low-density lipoprotein

cholesterol in the blood (Strazdiņa et al., 2013). There was a similar finding that the total SFA content in wild sandworms was higher than that in cultured sandworms, and the reasons for fatty acid difference were related to food sources and living conditions (Ahmad et al., 2015).

People in South China like to eat the game, including wild bullfrogs, due to its high nutritional value and delicious taste; nevertheless, research on nutrition and risk of fatty acids of wild and cultured bullfrogs to human health is still scarce. The *R* value represents the diversity in fatty acid levels, which was defined as the ratio of fatty acid concentrations in wild and cultured bullfrogs (Fig. 4a). *R* values are good for comparing the health risk between wild and cultured bullfrogs; moreover, we can rapidly screen for healthier and less toxic food for people. In the muscle, the *R* values of PUFA (n-3) and PUFA (n-6) were higher than 1, which indicated that wild bullfrogs had higher levels of PUFA (n-3) and PUFA (n-6) than cultured bullfrogs. PUFAs (n-3), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can maintain the balance of cell factor and lipoprotein, accelerate growth and be beneficial to the treatment of coronavirus disease (COVID-19) (Djuricic and Calder, 2021). Low AI values were regarded as a healthy diet and of benefit to retard atherosclerosis and decrease the risk of cardiovascular disorders (Zula et al., 2021; Bland et al., 2021; Ulbricht and Southgate, 1991). The AI of the muscle in wild bullfrogs was higher than that in cultured bullfrogs, indicating that people had higher risks of cardiovascular disorders when they consumed wild bullfrogs instead of cultured bullfrogs. In addition, a similar trend was observed that the *R* value of AI in whole bullfrogs was greater than 1 (Fig. 4c). Altogether, people should pay more attention to the health risks of game food instead of cultured food.

3.4.2. *Effects of different cooking methods*

A few changes occur in cooking processes; specifically, lipid oxidation, diffusion and exchange contribute to relative changes in fatty acids, such as SFAs, PUFAs, and monounsaturated fatty acids (MUFAs), resulting in changes in individual PFASs concentrations (Fig. 6). The use of nonparametric Spearman's rho rank-order correlation analysis allows to obtain more statistically significant information on the sources of fatty acids and PFASs. There was a statistically significant inverse correlation between ∑PUFAs and PFASs ($R = -0.90$, $p < 0.05$) (Table S5). In the steaming method, different classes of PFASs concentrations were at low levels; in contrast, different classes of fatty acids were at high levels (Fig. 6), which was similar to the results in different tissues of bullfrogs found in this study. These results were related to the above conclusion that PFASs bound FABP and competed with fatty acids. Previous results based on the fluorescence displacement assay have already shown that the FABP binding affinity of PFASs was clearly increased as carbon chain increased to C4-C11 and was reduced when the carbon chain was more than C11(Zhang et al., 2013).

Total fatty acid concentrations were higher in pan-fried and deep-fried bullfrogs than in raw bullfrogs because fatty acids may be at a high concentration in the oil and transferred into the bullfrog samples. NVI, as a ratio of the nutritive value index, was assessed in the raw and cooked bullfrogs. Cooked bullfrogs had higher NVI values than raw bullfrogs, which meant that cooked bullfrogs had high nutritive values. Similar to our findings, the NVI values of cooked rabbit meats were higher in samples prepared by different cooking methods than in raw samples (Abdel-Naeem et al., 2021).

The compositions and concentrations of fatty acids had a maximum degree of variation, and the PUFA concentration in the steaming process was higher than that in the other cooking methods. SFA concentrations were lower than PUFA concentrations in raw bullfrogs, but it is worth noting that SFA concentrations increased and were even higher than PUFA concentrations by most cooking methods except deep-frying at 170 ℃. On the one hand, the increment of SFA was observed in samples by different cooking methods because PUFA can be converted into SFA

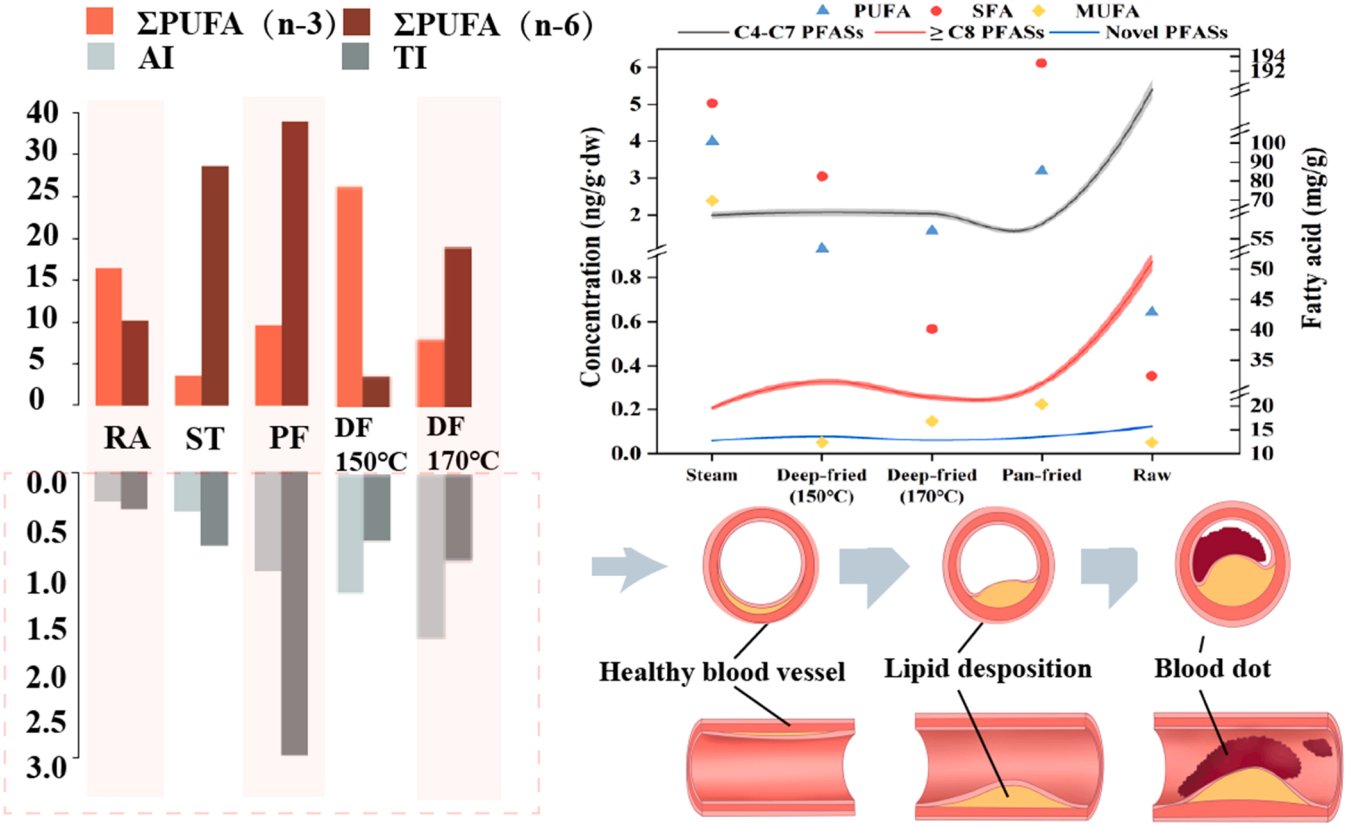

Fig. 6. Correlation between fatty acids and PFASs in bullfrogs by different cooking processes **Note:** Values on the left and right sides represent PFAS concentrations and fatty acid concentrations in each picture.

(Larsen et al., 2010). On the other hand, PUFAs have a lower melting point than SFAs, and PUFAs (including the original fatty acids of samples and the oil) have a stronger tendency for degradation than SFAs. Food with a PUFA/SFA proportion of over 0.45 was proposed as a healthy food to reduce the incidence of cancer and other diseases such as cardiovascular diseases (Werenska et al., 2021). Out of all cooking processes, only the pan-fried process had a low PUFA/SFA ratio, even below 0.45, which implied that it would induce a cholesterol increase in blood resulting in disease.

Essential fatty acids are important for humans because they cannot be synthesized in the body and need to be obtained through dietary supplementation. PUFA (n-3) and PUFA (n-6), as essential fatty acids, are regarded as highly valuable nutritional fatty acids owing to their advantages of lowering susceptibility to mental diseases and alleviating rheumatoid arthritis symptoms (Zula et al., 2021). PUFA (n-3) was at high levels after being deep-fried at 150 °C, and a considerable amount of PUFA (n-6) was detected in steaming and pan-fried processes. The AI and TI values were more than 1 in the frying processes, including pan-frying (1.05), deep-frying 150 ℃ (1.19) and deep-frying at 170 ℃ (1.68), as shown in Table 1. Compared with different cooking methods, the AI of steamed bullfrogs was the lowest, reaching 0.42. This means that consuming steamed food does not increase the level of blood cholesterol and reduce low-density lipoprotein cholesterol; thus, foods cooked by this method do not pose risk of arteriosclerosis; conversely, other cooking methods have higher health risks than steaming. Of note, steaming, deep-frying, and pan-frying processes are considered to be effective cooking methods for reducing the content of PFASs in bullfrogs. Steaming is particularly recommended as the preferred cooking method for reducing health risks caused by PFASs, while maintaining a high nutritional value, and has less disease risk for arteriosclerosis. Thus, the harmfulness of AI, TI, and PFASs concentrations in bullfrogs should not be ignored when considering the food safety of bullfrogs.

4. Conclusions

Bullfrogs, as indicator species used to assess ecosystem and human health risks, were investigated (different tissues of bullfrogs) by various cooking methods. Of note, the highest TMR values of each pair of similar carbon chains were in the same tissue of wild bullfrogs. Fat, protein and fatty acids may affect the accumulation of PFASs in different tissues and by different cooking methods of wild and cultured bullfrogs. There was a statistically significant correlation between nutrients and PFASs in bullfrogs.

The AI value was higher in the muscle of wild bullfrogs than in the muscle of cultured bullfrogs, which means that people have a higher risk of cardiovascular disorders when they consume wild bullfrogs instead of cultured bullfrogs. Novel PFASs (e.g., ADONA, PFMOPrA, PFMOBA and F-53B) had a higher detection level and concentration in wild bullfrogs than in cultured bullfrogs. The pollutant concentrations can increase by consuming wild bullfrogs; this should be taken seriously, and control measures should be taken to reduce the consumption of wild bullfrogs.

∑PFASs concentrations in bullfrogs were reduced for each cooking method. When comparing different cooking methods in this study, it was determined that the most effective cooking method for reducing PFASs concentrations was the steaming. Based on the high loss ratio of PFASs, high nutritive value and low disease risk in biological media, this study recommends the steaming method for cooking bullfrogs.

Environmental implication

Bullfrogs have unique ecological functions in aquatic and terrestrial environments and can be used as an indicator for contaminant levels. This is the first report on novel PFASs (e.g. F-53B and PFMOBA) and nutritional values in wild/cultured bullfrogs after cooking. Meanwhile, potentially confounding factors that change PFASs were explored. Higher levels of PFASs were found in wild bullfrogs than in cultured; however, PFASs concentrations were reduced after being cooked. Steamed bullfrogs are recommended in terms of risk-benefit ratio balance. This study provides baseline information on PFASs and nutrients in bullfrogs and raises awareness for avoiding adverse effects from bullfrog consumption.

Table 1
Fatty acid concentrations (mg/g·dw) and health index in bullfrogs.

	Item	ΣPUFA	ΣPUFA(n-3)	ΣPUFA(n-6)	EPA+DHA	SFA	ΣMUFA	AI	TI	PI	NVI
Cultured	Intestines	42.42	20.31	19.98	20.31	12.62	23.43	1.67	0.07	/	/
	Stomach	54.57	12.26	32.32	12.26	13.54	34.48	0.71	0.09	/	/
	Skin	62.25	14.93	30.91	14.93	31.99	31.47	0.83	0.39	/	/
	Muscle	44.57	13.06	11.96	13.06	43.67	12.51	0.71	0.81	/	/
	Liver	38.09	30.44	3.13	30.44	33.84	7.44	0.09	0.25	/	/
Wild	Intestines	71.68	42.20	21.77	42.20	26.82	27.25	0.20	0.08	/	/
	Stomach	14.04	0.45	7.92	0.45	6.85	7.53	0.29	0.25	/	/
	Skin	60.04	33.57	11.22	33.57	29.71	15.38	0.23	0.12	/	/
	Muscle	136.89	26.89	72.40	26.89	50.9	73.32	0.86	0.31	/	/
	Liver	119.44	9.72	9.65	9.72	86.51	15.73	0.05	1.60	/	/
Cooked	Raw	42.92	16.47	10.20	16.47	32.35	12.33	0.32	0.40	3.33	2.28
	Pan-fried	85.24	3.62	28.74	3.62	193.06	20.33	1.05	3.00	0.70	20.30
	Steaming	100.98	9.62	34.05	9.62	123.45	69.68	0.42	0.79	1.21	7.94
	Deep-fried 150°C	53.40	25.47	3.67	25.47	82.44	12.35	1.19	0.67	2.69	7.10
	Deep-fried 170°C	56.30	7.81	18.55	7.81	40.09	16.75	1.68	0.88	0.32	4.40

CRediT authorship contribution statement

Qiongping Sun: Investigation, Methodology, Formal analysis, Data curation, Writing – review & editing. **Jiaer Zhang:** Investigation, Formal analysis, Data curation, Writing – review & editing. **Tieyu Wang:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration. **Yonglong Xiong:** Investigation, Formal analysis, Data curation, Writing – review & editing. **Xinyi Zhan:** Investigation, Formal analysis, Data curation, Writing – review & editing. **Hancheng Zhao:** Investigation, Formal analysis, Data curation, Writing – review & editing. **Jianwen Wang:** Investigation, Formal analysis, Data curation, Writing – review & editing. **Yueyao Fan:** Investigation, Formal analysis, Data curation, Writing – review & editing. **Ran Bi:** Investigation, Formal analysis. **Shuqi Wang:** Investigation, Formal analysis. **Seongjin Hong:** Conceptualization, Writing – review & editing. **Jong Seong Khim:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.130555.

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Supplementary Material

Cooking Methods Effectively Alter Perfluoroalkyl Substances and Nutrients in Cultured and Wild Bullfrogs

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Content:

Extraction and Cleanup

The extraction of samples was used as a previous method (Diao et al. 2022; Sun et al. 2021). First, 1 g of sample and 5 ng of internal standards (MPFAC-MXA) were thoroughly mixed in a polypropylene (PP) tube. 1 mL 0.5M tetrabutylammonium hydrogensulfate solution and 2 mL of 0.25M sodium carbonate buffer (pH 10) were added into a 50 mL PP centrifuge tube for extraction. After mixing, add 5 mL of methyl-tert-butyl ether (MTBE) and shake the mixture at 250 rpm for 15 min. The organic and aqueous layers were separated by centrifugation at 3000 rpm for 10 min. Then 5 mL of MTBE was taken and transferred to a new 50 mL PP tube, and the extraction was repeated twice as above. All extracts were combined in the same new 15 mL PP tube. After added 1 mL of methanol, the final extract was evaporated to 1 mL with a gentle stream of high purity nitrogen. The 1 mL extracts were further purified by using ENVI-Carb and solid phase extraction (SPE) cartridges. The ENVI-Carb cartridge was purified with 1 mL menthol 3 times. 1 mL extracts flowed through the cartridge and collected. Then 1 mL menthol was used to wash the PP tube and cartridge three times, respectively.

Totally, 7 mL elution was collected and transferred to PP tube, diluted to 100 mL with Milli-Q water and extracted with the OASIS WAX-SPE cartridge. The SPE cartridge was preconditioned with 4 mL of 0.1% NH_4OH in methanol, 4 mL methanol and 4 mL Milli-Q water. Then 100 mL sample was loaded into the cartridge. The cartridge was washed with 20 mL Milli-Q water, 4 mL of 25 mM ammonium acetate, allowed to run dry, and eluted with 4 mL methanol and 4 mL of 0.1% ammonia in methanol. The eluents were concentrated to 0.5 mL under high purity nitrogen and passed through a nylon filter (0.2 μm), then transferred into a 1.5 mL auto-sampler vial fitted with PP cap for HPLC analysis.

Table S1. Chemical formula, parent ion, quantitative ion and qualitative ion of PFASs

Analyte	Compound	Chemical formula	Parent Ion (m/z)	Quantitative ion (m/z)	Qualitative ion (m/z)
PFCAs					
PFBA	perfluoro-butanoate acid	$C_3F_7CO_2^-$	212.9	169.0	195.3
PFPeA	perfluoropentanoate acid	$C_4F_9CO_2^-$	262.9	218.9	141.0
PFHxA	perfluorohexanoate acid	$C_5F_{11}CO_2^-$	312.9	268.9	119.1
PFHpA	perfluoroheptanoate acid	$C_6F_{13}CO_2^-$	362.8	318.9	169.0
PFOA	perfluorooctanoate acid	$C_7F_{15}CO_2^-$	412.8	368.9	169.0
PFNA	perfluorononanoate acid	$C_8F_{17}CO_2^-$	462.8	418.9	218.9
PFDA	perfluorodecanoate acid	$C_9F_{19}CO_2^-$	512.8	468.9	218.7
PFUndA	perfluoroundecanoate acid	$C_{10}F_{21}CO_2^-$	562.9	518.9	268.9
PFDoDA	perfluorododecanoate acid	$C_{11}F_{23}CO_2^-$	612.8	568.9	318.9
PFTrDA	perfluorotridecanoate acid	$C_{12}F_{25}CO_2^-$	662.8	618.9	318.9
PFTeDA	perfluorotetradecanoate acid	$C_{13}F_{27}CO_2^-$	712.8	668.9	368.9
PFHxDA	perfluorohexadecanoate acid	$C_{15}F_{31}CO_2^-$	812.8	768.8	368.9
PFODA	perfluorooctadecanoate acid	$C17F_{35}CO_2^-$	912.8	368.9	568.9
PFSAs					
PFBS	perfluorobutane sulfonate	$C_4F_9SO_3^-$	298.8	98.9	80.0
PFHxS	perfluorohexane sulfonate	$C_6F_{13}SO_3^-$	399.0	98.9	80.0
PFOS	perfluorooctane sulfonate	$C_8F_{17}SO_3^-$	498.9	99.0	80.0

PFDS	perfluorodecane sulfonate	$C_{10}F_{19}SO_3^-$	598.8	98.9	79.8
F-53B	chlorinated polyfluoroalkyl ether sulfonic acid	9CL-PF_3ONS	530.9	350.9	82.9
ADONA	ammonium 4,8-dioxa-3Hperfluorononanoate	$C_7F_{12}O_4H^-$	376.0	251.1	85.2
PFMOPrA	perfluoro-4-oxapentanoic acid	$C_4F_7O_3H^-$	228.9	85.1	185.2
PFMOBA	perfluoro-5-oxahexanoic acid	$C_5F_9O_3H^-$	278.9	85.3	234.8
$^{13}C_4$ PFBA	$^{13}C_4$Perfluoro-butanoic acid		217.0	172.0	185.0
$^{13}C_4$PFHxA	$^{13}C_4$Perfluoro-hexanoic acid		314.9	255.2	296.9
$^{13}C_4$ PFOA	$^{13}C_4$Perfluoro-octanoic acid		416.8	371.9	168.9
$^{13}C_4$ PFNA	$^{13}C_4$Perfluoro-nonanoic acid		467.8	422.9	219.0
$^{13}C_4$ PFDA	$^{13}C_4$Perfluoro-decanoic acid		514.8	469.9	268.9
$^{13}C_4$PFUDA	$^{13}C_4$Perfluoro-undecanoicacid		564.8	519.9	268.9
$^{13}C_2$PFDoA	$^{13}C_4$Perfluoro-dodecanoicacid		614.8	569.9	270.1
$^{18}O_2$ PFHxS	$^{18}O_2$Perfluorohexanesulfonate		402.8	84.1	103.0
$^{13}C_4$ PFOS	$^{13}C_4$Perfluoro-octanesulfonate		502.8	104.2	80.0

Table S2. HPLC-ESI-MS instrument conditions

HPLC conditions		
analytical column	Aglient ZORBAX Eclipse Plus C18, 2.1×100 mm, 3.5 μm	
guard column	Agilent 1290 Infinity In-line filter with 0.3 μm SS frit	
injection volume	5 μL	
column temperature	40 °C	
mobile phase	A = 2 mM ammonium acetate	
	B = 100% Acetonitrile	
run time	16 min + 4 min post time	
flow rate	0.3 mL/min	
gradient	Time (min)	Mobile phase
	0	20% B
	14	90% B
	16	90% B
MS conditions		
acquisition parameters	ESI mode, negative ionization; MRM	
source gas temperature	350 °C	
source gas flow rate	9 L/min	
nebulizer pressure	40 psi	
capiliary	3500 V positive; 4000 V negative	
delta EMV(-)	200-400 V	

Table S3. GC-MS instrument conditions

GC conditions		
analytical column	Shimadzu GCMS-QP2010SE, 30 m× 0.25 mm× 0.25μm	
injection volume	1.0μL	
column temperature	250 °C	
instrument pressure	112.5 kPa	
split ratio	1.0	
gradient	retention time (min)	temperature (℃)
	5	100
	1	180
	2	220
	5	230
	3	250
MS conditions		
acquisition parameters	EI mode; Scan	
source gas temperature	325°C	
source gas flow rate	9 L/min	
nebulizer pressure	40 psi	
mass sweep range	35-600 m/z	

Table S4. Quality assurance and quality control (QA/QC) for PFASs in this study

PFASs	LOD[a] (ng/g)	LOQ[b] (ng/g)	Procedure blanks	Solvent blanks	Recovery
PFBA	5.35E-03	1.79E-02	<5.35E-03	<5.35E-03	131%
PFPeA	6.25E-03	2.09E-02	<6.25E-03	<6.25E-03	110%
PFHxA	1.34E-03	4.47E-03	<1.34E-03	<1.34E-03	90%
PFBS	4.69E-03	1.57E-02	<4.69E-03	<4.69E-03	91%
PFHpA	1.21E-03	4.03E-03	<1.21E-03	<1.21E-03	82%
PFOA	6.80E-03	2.28E-02	<6.80E-03	<6.80E-03	113%
PFHxS	1.17E-02	3.91E-02	<1.17E-02	<1.17E-02	91%
PFNA	4.69E-03	1.57E-02	<4.69E-03	<4.69E-03	100%
PFDA	6.80E-03	2.28E-02	<6.80E-03	<6.80E-03	99%
PFOS	3.26E-03	1.09E-02	<3.26E-03	<3.26E-03	100%
PFUdA	5.75E-04	1.91E-03	<5.75E-04	<5.75E-04	82%
F-53B	7.45E-04	2.48E-03	<7.45E-04	<7.45E-04	93%
PFDoA	1.21E-03	4.03E-03	<1.21E-03	<1.21E-03	97%
PFDS	5.00E-03	1.67E-02	<5.35E-03	<5.35E-03	91%
PFTrDA	5.00E-03	1.67E-02	<6.25E-03	<6.25E-03	98%
PFTeDA	1.17E-03	3.91E-03	<1.34E-03	<1.34E-03	118%
PFHxDA	1.57E-03	5.20E-03	<4.69E-03	<4.69E-03	82%
PFODA	1.83E-03	6.10E-03	<1.21E-03	<1.21E-03	71%
PFMOPrA	5.75E-03	1.93E-02	<6.80E-03	<6.80E-03	58%

PFMOBA	5.35E-03	1.79E-02	<1.17E-02	<1.17E-02	78%
ADOHA	2.42E-03	8.05E-03	<4.69E-03	<4.69E-03	83%

Note: a, LOQ was defined as the minimum concentration of a substance that can be quantitatively measured in the specific product with an acceptable level of accuracy and precision (SNR=10:1); b, The LOD was defined as the amount of chemicals which could be detected in a given amount of samples after the entire method was performed (SNR=3:1)

Table S5. The correlation between PFASs and nutrient contents

	Fat	Protein	Ash	ΣPUFA	SFA
∑PFASs	**0.68**	-0.33	0.38	0.43	0.42
< PFASs	-0.30	0.11	0.05	0.42	**0.64**
≥C8 PFASs	0.10	0.30	0.30	**-0.90**	-0.50

Note: Marked correlations (in bold) are statistically significant at the 95% confidence interval (p<0.05)

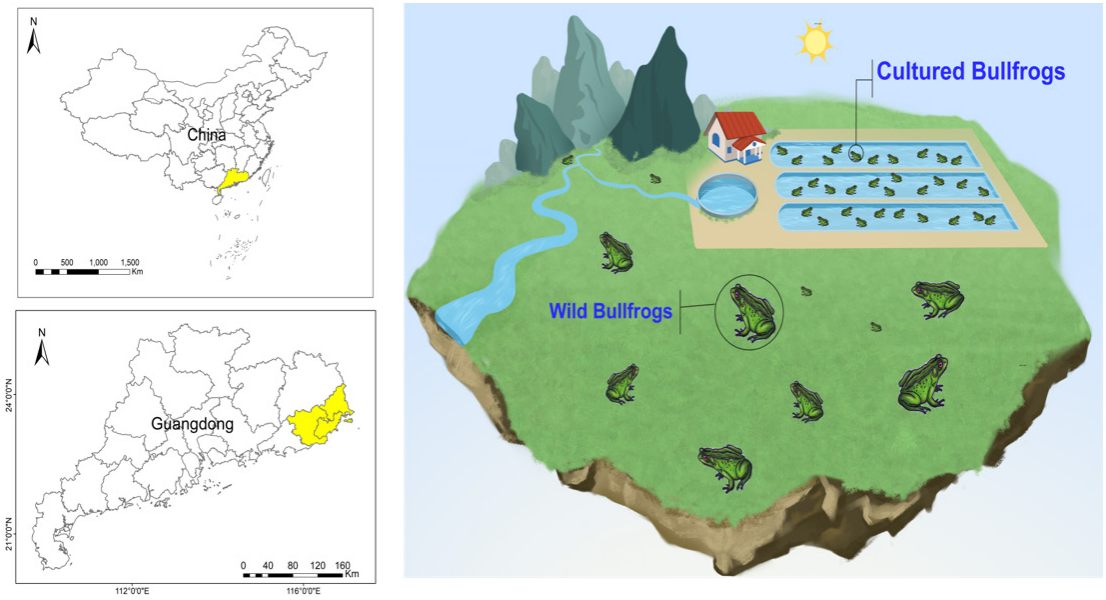


Figure S1. The sample sites of bullfrog farms

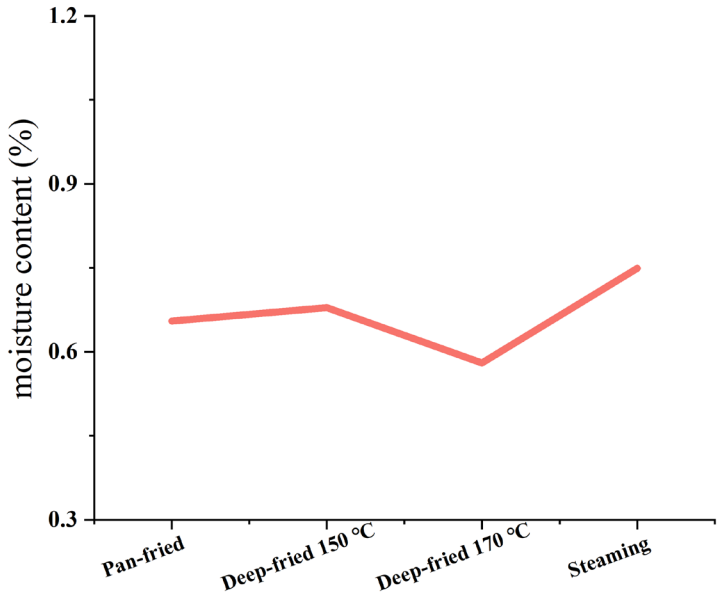


Figure S2. The moisture content in bullfrogs

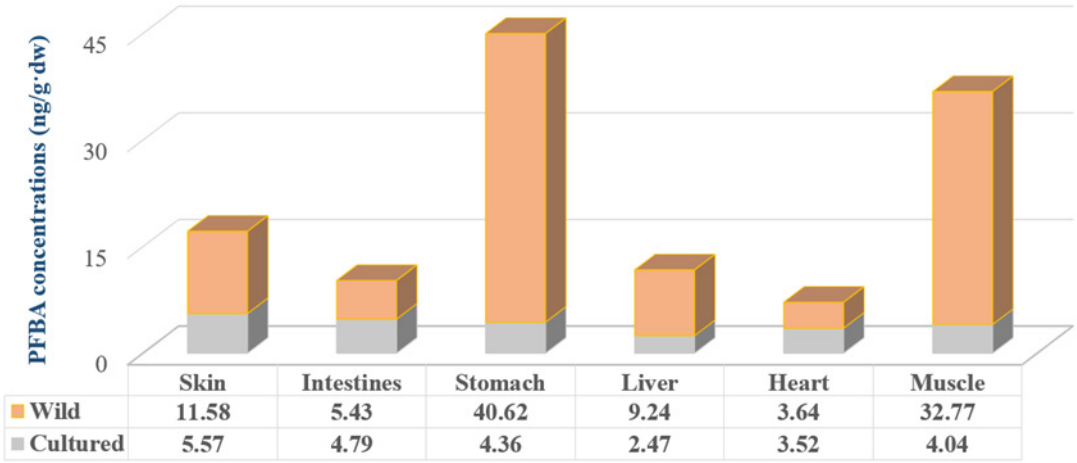


Figure S3. Mean PFBA concentrations in various tissues of bullfrogs (ng/g·dw)

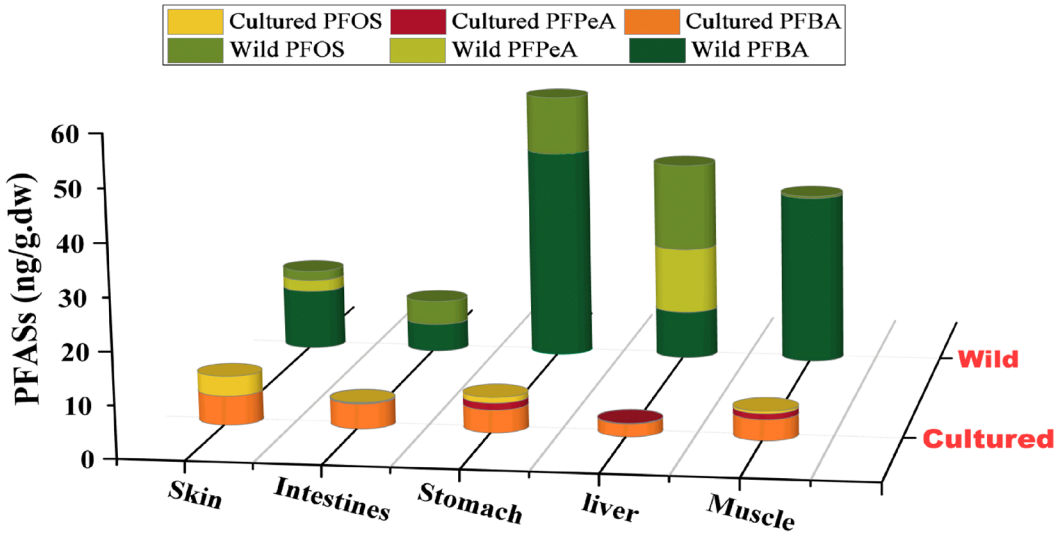


Figure S4. Traditional PFASs concentrations such as PFBA, PFPeA and PFOS in tissues of bullfrogs (ng/g·dw)

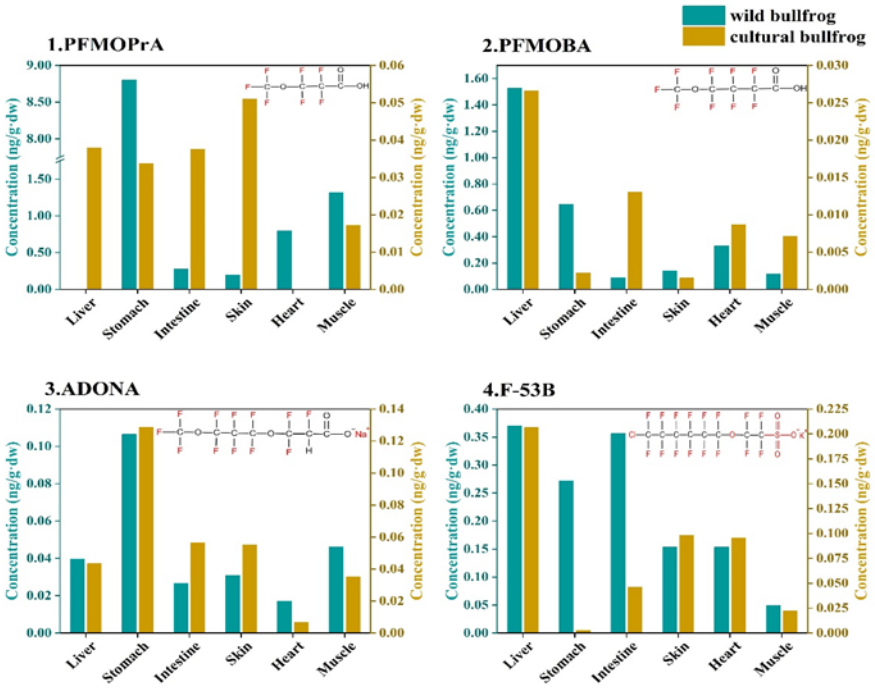


Figure S5. Novel PFASs concentrations in tissues of bullfrogs (ng/g·dw)

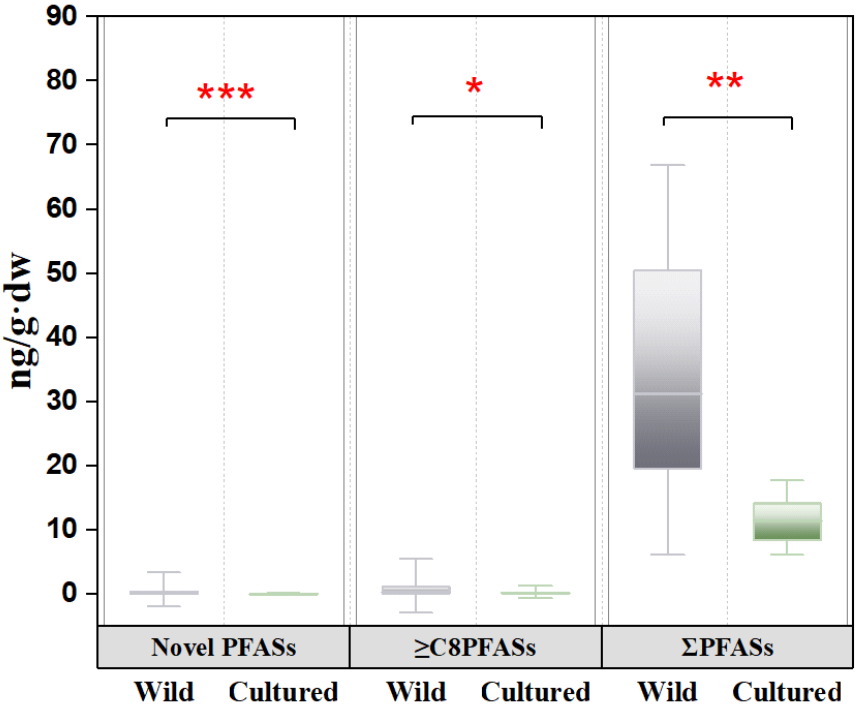


Figure S6. PFASs concentrations in wild and cultured bullfrogs (ng/g·dw)

Note: ★ represents the degree of significant difference. ★★★ means *p* is less than 0.001; ★★ shows *p* is less than 0.01; ★ describes *p* is less than 0.05. The box ranged from 25% to 75%. Line represents standard deviation (SD). The medians were shown in middle position.

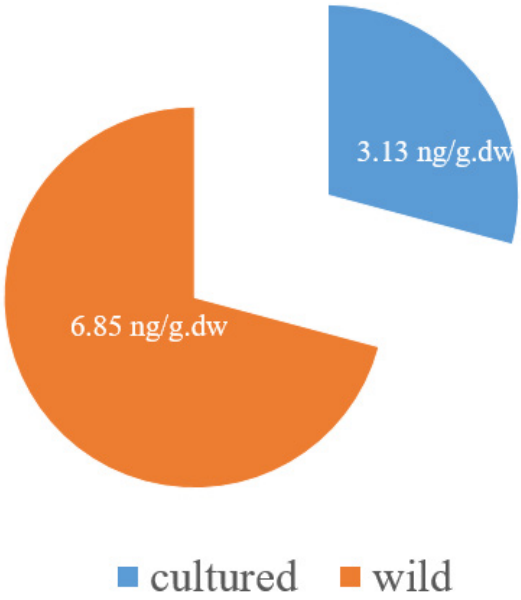


Figure S7. The comparison of mean PFOS concentration (ng/g·dw) between cultured and wild bullfrogs

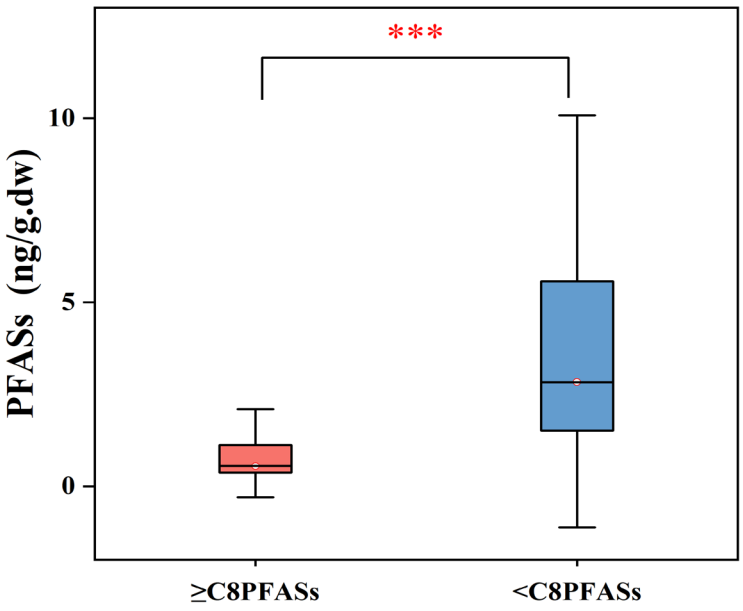


Figure S8. The comparison between ≥C8 PFASs and ＜C8 PFASs in bullfrogs

Note: ★★★ means p is less than 0.001. The box ranged from 25% to 75%. Line represents standard deviation (SD). The medians were shown in middle position.

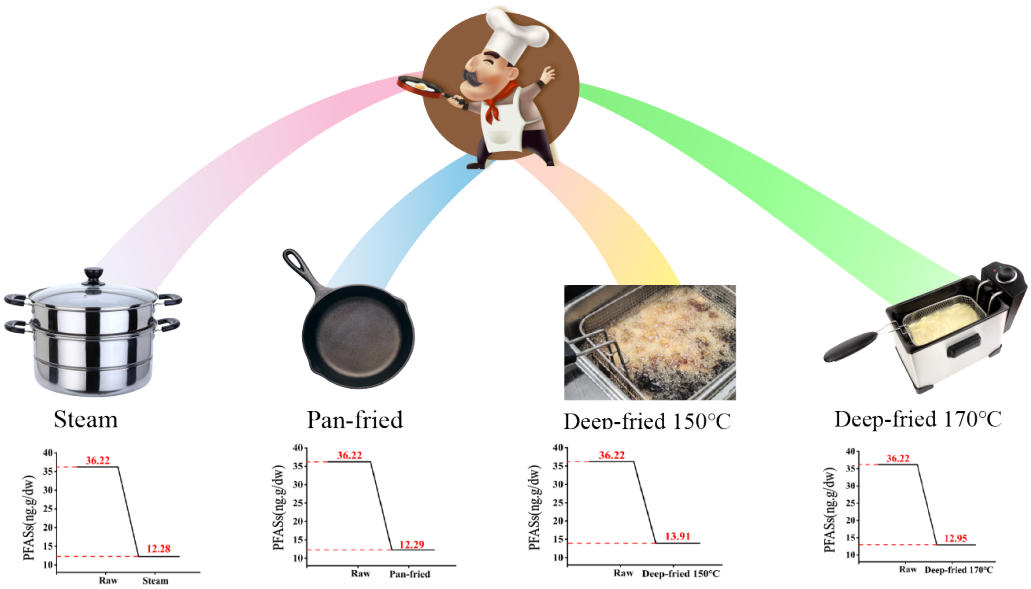


Figure S9. ΣPFASs concentrations (ng/g·dw) after different cooking methods

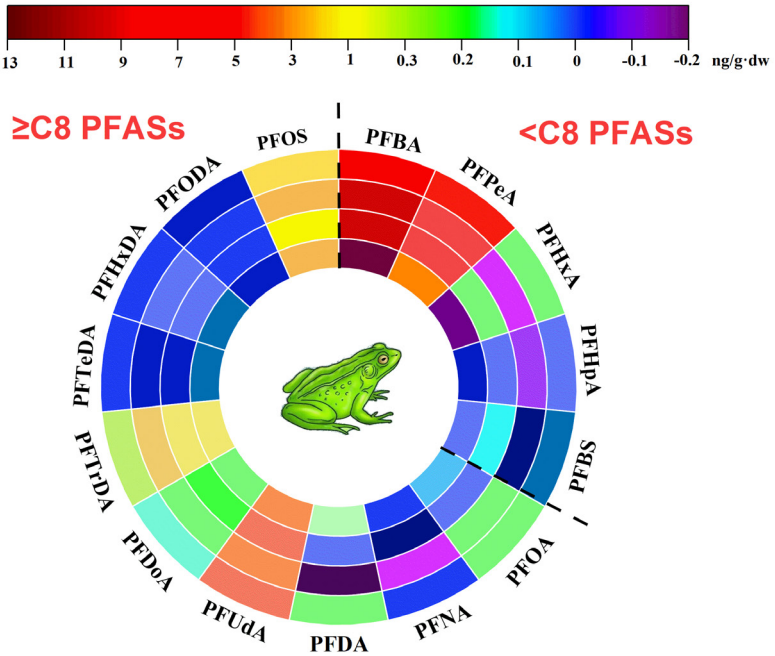


Figure S10. Loss of PFASs after different cooking methods (ng/g·dw)

Note: Circles represent steaming, deep-fried 150℃, deep-fried 170℃ and pan-fried from the inner circle to the outer circle, respectively.

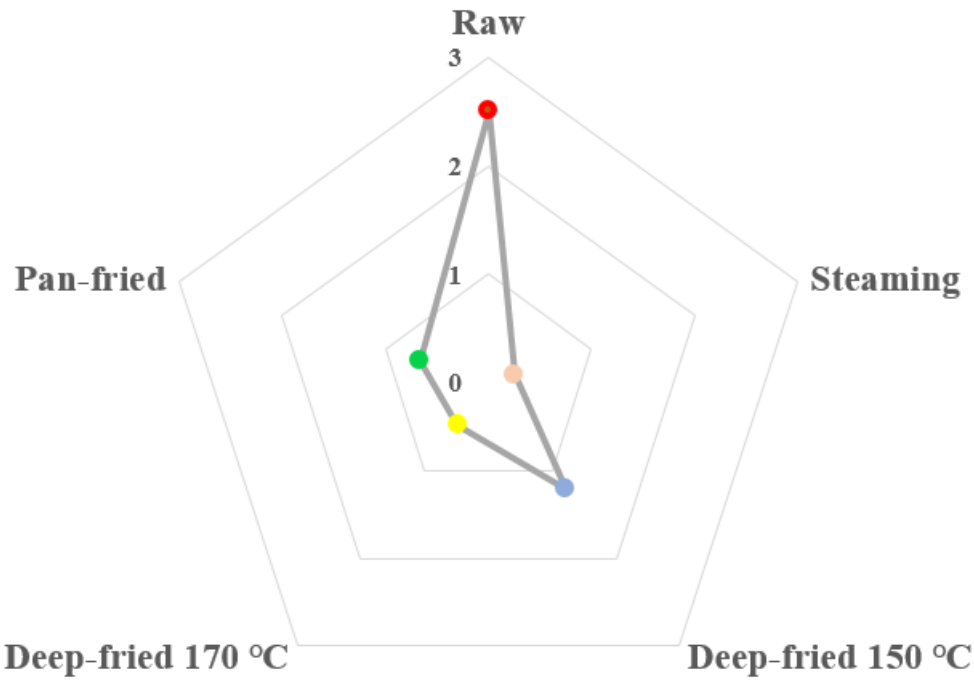
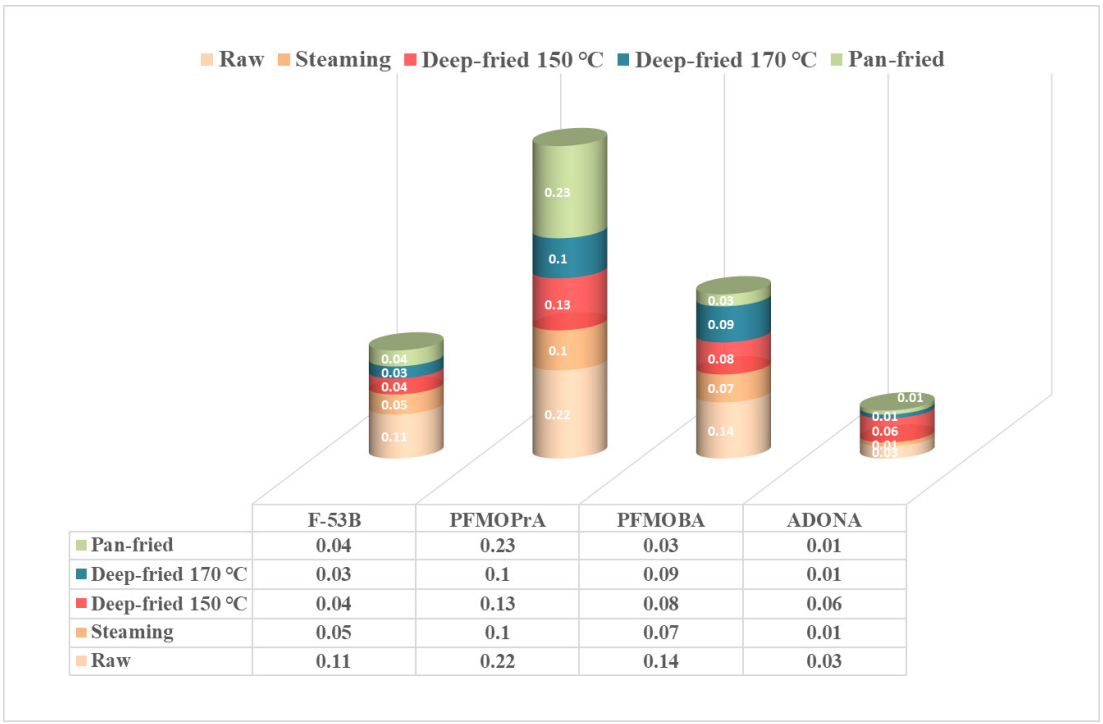


Figure S11. PFOS concentration change by different cooking methods (ng/g·dw)



	F-53B	PFMOPrA	PFMOBA	ADONA
Pan-fried	0.04	0.23	0.03	0.01
Deep-fried 170 °C	0.03	0.1	0.09	0.01
Deep-fried 150 °C	0.04	0.13	0.08	0.06
Steaming	0.05	0.1	0.07	0.01
Raw	0.11	0.22	0.14	0.03

Figure S12. The variation of novel PFASs concentration by different cooking methods. (ng/g·dw)

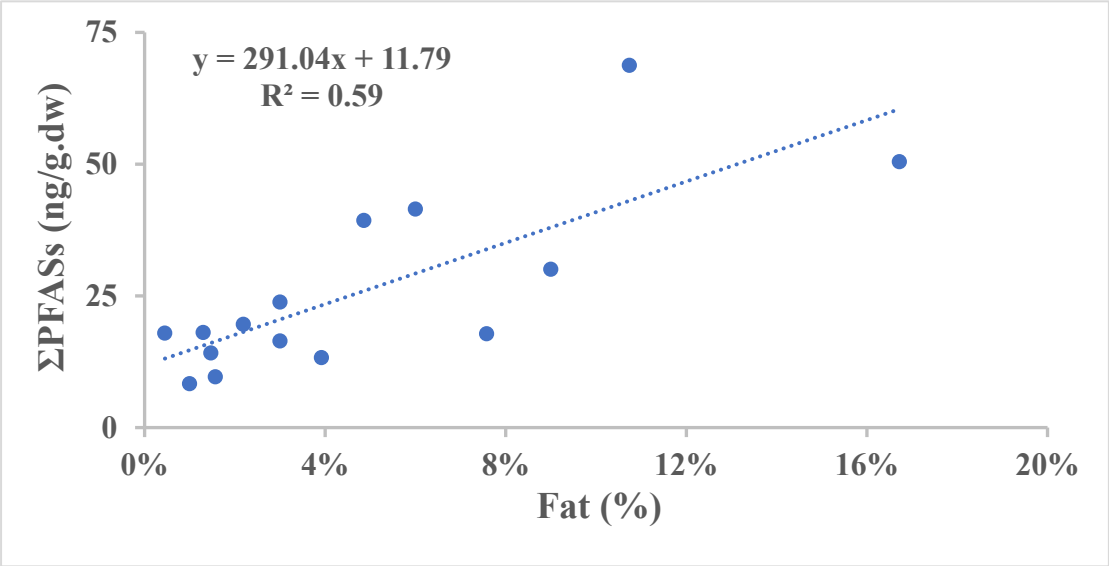


Figure S13. Linear correlation between fat (%) and Σ PFASs (ng/g·dw)

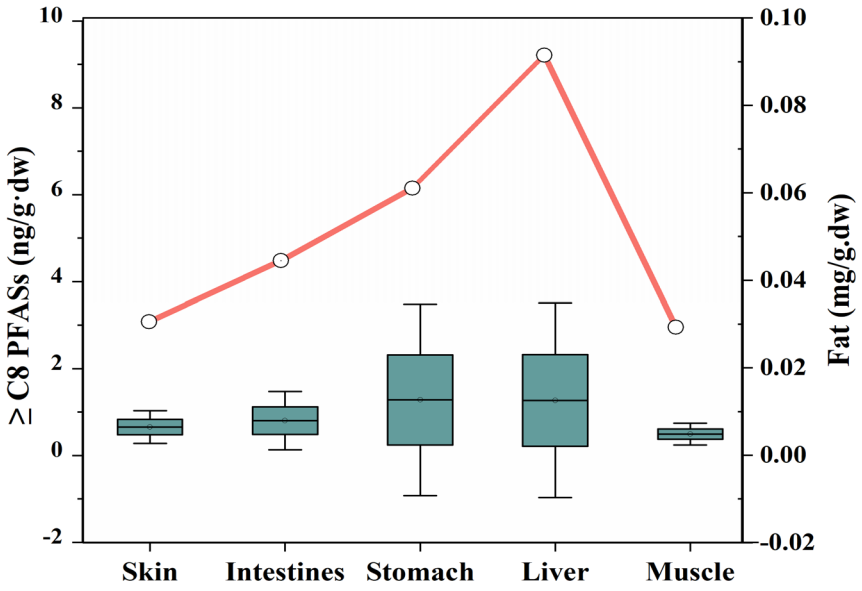


Figure S14. ≥C8 PFASs and fat concentrations in bullfrogs (ng/g·dw)

Note: The bar graph was used to express the tendency of ≥C8 PFASs concentration in different tissues; the curve represented the variation of fat in different tissues.

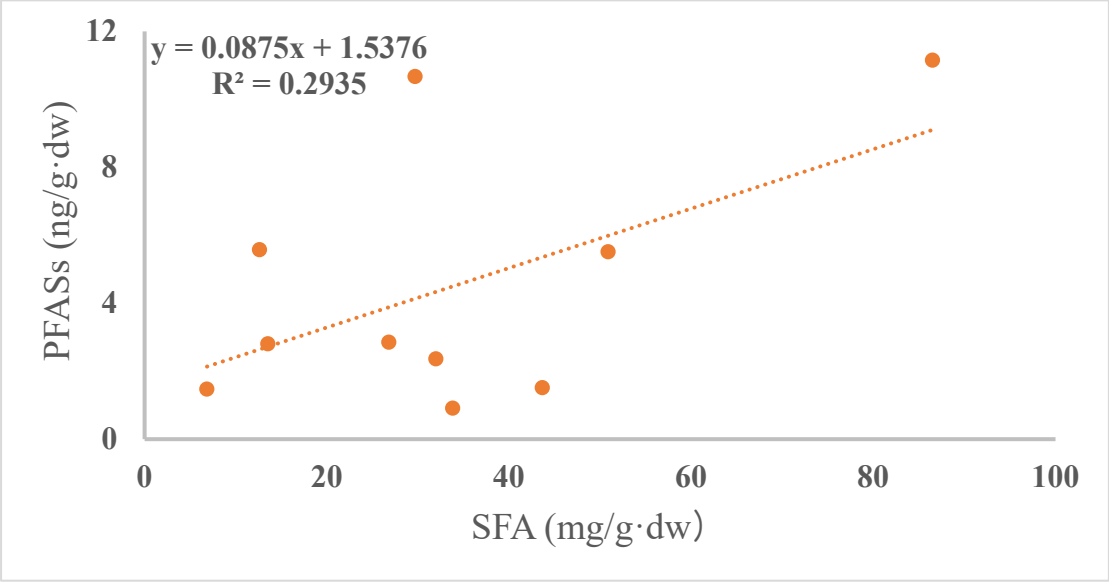


Figure S15. Linear correlation between SFA (mg/g.dw) and C4-C7 PFASs (ng/g·dw)

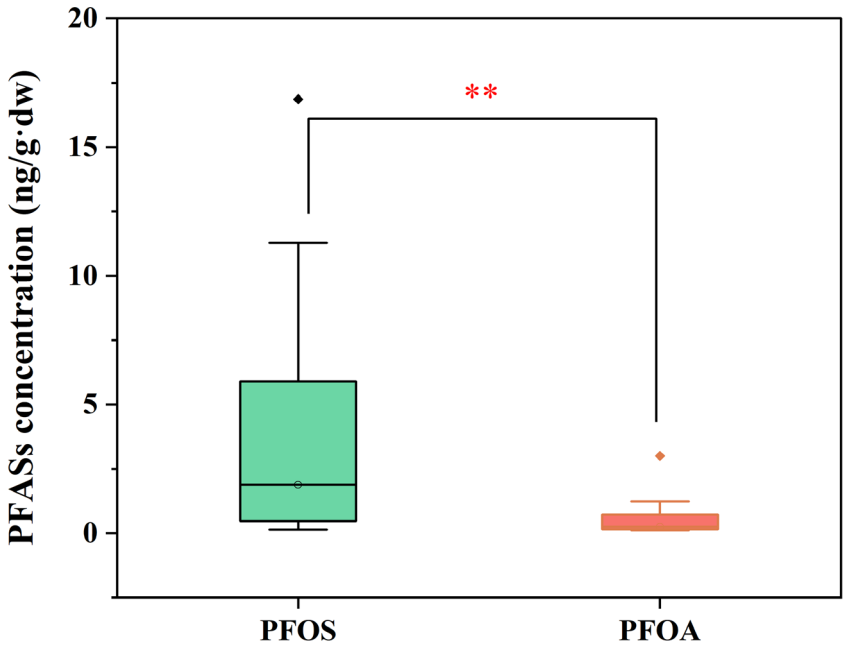


Figure S16. The comparison between PFOA and PFOS in bullfrogs

Note: ★★ means *p* was less than 0.01. The box ranged from 25% to 75%. Line represents standard deviation (SD). The medians were shown in middle position. Dots represented outliers.

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