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Minmin Li [a,1], Lin Li [a,1], Zhiqiang Kong [b], Noel Gregoire [c], Rui Quan [a], Zisheng Luo [d], Xingyu Lin [d], Jesus Simal-Gandara [e,*], Bei Fan [a,*], Fengzhong Wang [a,*]

[1] These authors contributed equally to this paper.

[a] Key Laboratory of Agro-products Quality and Safety Control in Storage and Transport Process, Ministry of Agriculture and Rural Affairs/Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences, Beijing 100193, P. R. China

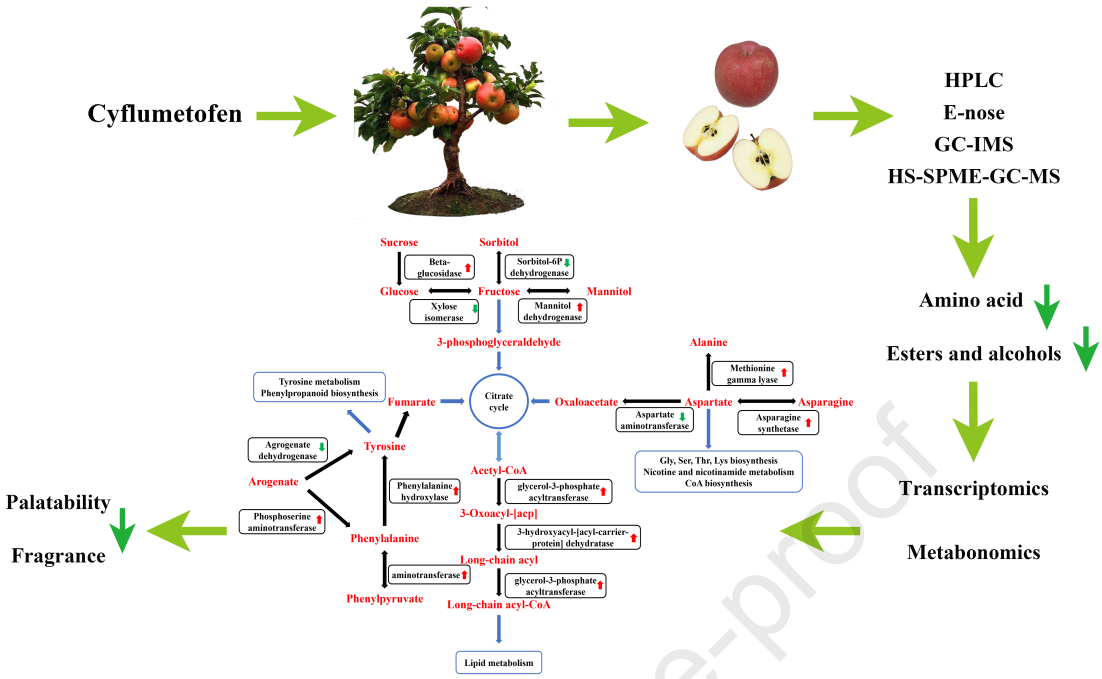
[b] State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, P. R. China

[c] Functional and Evolutionary Entomology, Gembloux Agro-Bio-Tech, University of Liège, Passage des Déportés 2, 5030 Gembloux, Belgium

[d] Zhejiang University, College of Biosystems Engineering and Food Science, Key Laboratory of Agro-Products Postharvest Handling of Ministry of Agriculture and Rural Affairs, Zhejiang Key Laboratory for Agri-Food Processing, Hangzhou, 310058, People's Republic of China

[e] Universidade de Vigo, Nutrition and Bromatology Group, Analytical Chemistry and Food Science Department, Faculty of Science, E32004 Ourense, Spain

Corresponding author: Bei Fan: fanbei517@163.com; Fengzhong Wang: wangfengzhong@sina.com; Jesus Simal-Gandara: jsimal@uvigo.es



Abstract

Pesticide residues were found to interfere the nutrition and flavor of fruits. Apple flavor changes after pesticides application was investigated based on metabolic dynamics and underlying regulatory networks. In this study, cyflumetofen (CYF) systematically affected the nutrition and flavor formation on apple (*Malus pumila* Mill). CYF alters nutritional composition, but not total content of soluble sugars and organic acids. Palatability-related amino acid content decreased around 15% in CYF-treated apple. The contents of esters and alcohols responsible for fragrance and flavor decreased by approximately 10% in CYF-treated apples compared with controls. Non-target metabolomic and transcriptomic analysis showed that CYF mainly affected amino acid-, organic acid-, polyphenol-, and lipid-metabolism related pathways, leading to altered nutritional and flavor characteristics. In conclusion, the results suggested that CYF affected the primary metabolism of apple, resulting in unpleasant changes in nutritional and flavor composition. This study provided new insight into the metabolic regulation of flavor after pesticides application.

Keywords: Cyflumetofen, Apple, Metabolome, Transcriptome, Flavor, Nutrition

1. Introduction

A proper daily apple intake improves the antioxidant parameters and promotes the accumulation of probiotics (Giarettaet al. 2019). Such a property made apple a popular fruit around the world. The flavor of apples is one of the most important quality indexes that influence consumer preference and market competitiveness. However, according to the USA Environment Working Group's shopper's guide (https://www.ewg.org/), for the past few years, apples have ranked among the 12 most contaminated fruits in the USA. According to the data from the China Apple Industry Association (CAIA), the annual planting area of apples in China was about 3.13 million hectares and the total production was about 4.41 million tons (CAIA 2021). To meet the need of healthy growth of apples, a lot of pesticides were applied in apple yards to deal with tens of insects and pathogenic microorganisms present over the lifespan of apples, among which, cyflumetofen (CYF), a benzoyl acetonitrile-based acaricide, has become a promising acaricide since its first registration in Japan in 2007. CYF is widely used due to its high selective efficacy against spider mite and its long persistence in the environment (Nobuyoshi, Hirofumi, & Yasuhiro, 2012). According to Guo et al. (2018), CYF was applied on apples two times with an interval of 10 days, and the initial residue of CYF enantiomers on apples was 470-480 μg/kg under 1.5-fold applied dosage, with a half-life of approximately 23 days.

Generally, pesticide residues would affect the flavor or nutrition formation of

agriculture production and result in decreased quality. For instance, fenhexamid increased post-harvest tomato weight-loss and reduced the quality of fruit appearance, while pyraclostrobin, together with boscalid, was found to reduce vitamin C content in tomato (Domínguez, Ferreres, & Pascual del Riquelme, 2012). Additionally, flavor variations in the finished products were notorious and directly influenced customer choice. Even under good agricultural practices (GAP), the application of pesticides reduced the terpene content in white grapes, resulting in decreased floral nuance in white wine (Gonzalez-Rodriguez, Noguerol-Pato, & Gonzalez-Barreiro, 2011). Pesticide residues significantly inhibited yeast growth during beer fermentation, leading to reduced content of isobutyl and isoamyl alcohols and increased ethyl-acetate content in beer (Kong 2016). However, it is still unclear whether these flavor and nutrition changes reduce or improve the quality of apples being treated with pesticides.

The domestic apple (*Malus pumila* Mill) flavor involves a complex set of interactions between taste and olfactory sensing, which is not always easy to measure quantitatively and highly affected by the environment (Tieman et al. 2017). The key genes involved in flavor formation and regulation have not been well defined. Over the past decades, integration of multi-omics data such as metabolomics and transcriptomics has been proven to be an excellent way to analyze flavor metabolic pathways and identify regulatory genes (Osorio et al. 2012). Brizzolara et al. (2017) used a metabolomics approach to identify the most vulnerable flavor composition in

apple, that were ethyl ester and 2-methylbutyl derivatives, during atmosphere-controlled storage. However, there has been little research on the integration of metabolomics and transcriptomics data to illustrate the mechanisms of flavor or nutrition variation throughout apple ripening.

Flavor compounds increase substantially during fruit ripening, taking place during 20 to 21 weeks of fruit development and caused by an autocatalytic burst of ethylene production in the same time, which is the characteristic of all climacteric fruit (Fellman, Miller, & Mattinson, 2000). In the same time, the photosynthate in apple that accumulated in the early time was transformed into sugars. In this study, we applied CYF on apple during this time to figure out if CYF will affect the and flavor and nutrition formation in apple and how CYF functioned in this process. We investigated the flavor and nutrition changes using combined gas chromatography-mass spectrometry (GC-MS) and microarray-based analysis towards apple during ripening period after CYF application. Thereafter, metabolomics and transcriptomics were applied to make a comprehensive analysis on the process that CYF functions on the flavor and nutrition formation in apple. This study paved the way for the elucidation of the molecular mechanism of the apple flavor and nutrition variations caused by pesticide application.

2. Material and methods

2.1. Chemicals and reagents

Cyflumetofen (20%, SC) was purchased from Jiangsu FMC Corporation (Jiangsu,

China). CYF (97%) was purchased from Otsuka AgriTechno Co., Ltd. (Osaka, Japan). Ultra-pure water was obtained using a Milli-Q ultra-pure water system (Millipore, Burlington, MA, USA). HPLC-grade isopropanol, methanol, and acetonitrile were purchased from Fisher Scientific (Shanghai, China). Other chemicals were purchased from Sinopharm (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). DL-2-octanol and n-alkanes (C10-C24, intermediate mixed in hexane, 1000 mg/L) were purchased from Yuanye (Shanghai yuanye Bio-Technology Co., Ltd, Shanghai, China).

2.2. Field trials and fruit sample collection

Field trials were conducted in a *Fuji* apple orchard in Changping District, Beijing (40°22'N, 116°23'E), with no history of CYF treatment. Spraying treatment was applied four times at 15-day intervals during the late expanding and ripening period (BBCH76-85, For pome fruit, 76 to 79 stands for fruit about 70% to 90% final size; 81 stands for beginning of ripening (first appearance of cultivar-specific color); 85 stands for advanced ripening (increase in intensity of cultivar-specific color) (Uwe, 2001), with 1.5× and 5× (0.20 and 0.67 mg/L of active ingredient) the CYF (20%, SC) recommended dose (RD). Three trial plots were set with a guard row between each of the two trial plots per treatment. In the CYF treatment, all apples were sprayed with 1.5× (CYF_1.5) or 5× (CYF_5) the CYF (20%, SC) RD. Apples in the control treatment were sprayed with water (CYF_CK). Samples were collected 2 h after the last spray. Intact mature apples of similar size and without disease or insect damage

were selected as the experimental material. All the collected apples were distributed uniformly in the canopy. There were two trees in each treatment. In order to minimize the nutrition and flavor difference between different trees, two additional trees were set considering light and position, which was divided into three parts and treated as CYF_CK, CYF_1.5 and CYF_5 and apples in the middle of each part were collected to avoid the contamination of CYF on control ones. Thus, there were totally 8 trees in this study. The apples from one tree were collected and stored separately under the same conditions. Four apples from different trees were collected as one replicate and there were generally three replicates in the following tests. There were totally about 120 apples collected for each treatment. The design for the whole test was shown in Fig. S1.

2.3. Detection of CYF deposition on apple fruits

All samples were chopped without cleaning, recollected by coning and quartering, and homogenized. The homogenate (10.00 ± 0.01 g) was mixed with water (5 mL) and acetonitrile (10 mL) and shaken for 5 min at 1200 rpm (SPEX Sample Prep, Metuchen, NJ, USA). Subsequently, the mixture was vortexed (XW-80A Vortex, Kirin Medical Instrument, Jiaxing, China) for 3 min with the addition of $MgSO_4$ (4.0 ± 0.01 g) and NaCl (1.0 ± 0.01 g). The mixture was then centrifuged for 5 min at 2100 *g* (TG16-WS, Xiangyi Centrifuge Machines, Changsha, China). The supernatant was vortexed in a sorbent tube for 2 min and centrifuged for 5 min at 2100 *g* to obtain the

final supernatant, which was filtered through a 0.22 μm organic nylon membrane for UPC^2-MS/MS analysis using previously described methods (Guo et al. 2018).

2.4. Detection of apple nutritional composition

All samples were homogenized and centrifuged at 4 °C and 2100 *g*. Soluble sugar (fructose, glucose, sucrose, and D-sorbitol) and organic acid (malic acid, tartaric acid, lactic acid, and citric acid) contents were determined by HPLC (Waters, MA, USA. CAPECELLPAK MGS5 C18, 4.6 mm × 250 mm, 5 μm) (Mikulic-Petkovsek, Schmitzer, & Slatnar, 2012). The amino acid content was determined using a high-speed amino acid analyzer (Hitachi L-8800, Tokyo, Japan) (Ayalew, Retta, & Desse 2017).

2.5. Detection of apple flavor

2.5.1. Apple flavor detection using E-nose

Apple flavor was detected by Heracles II (Alpha-Mos Company, Toulouse, France) according to the method of Śliwińska Wiśniewska, & Dymerski, (2016) with some modifications. Apple slices (1.00 ± 0.01 g) were placed in a 20-mL headspace glass vial and incubated at 50 °C for 30 min with agitation at 5000 rpm. Thereafter, the headspace (3000 μL) was transferred to the injector at 200 °C, at an injection rate of 125 μL/s. The initial temperature for chromatography (MTX-5 with 5% diphenyl and MXT1701 with 14% cyanopropyl phenyl, 10 m × 0.18 mm × 0.4 μm) was 70 °C for 18 s; it was then increased to 240 °C in 85 s and sustained for 30 s. The detector temperature was set at 260 °C. Clean air was used as the carrier gas at a flow rate of

10 mL/min. The whole Heracles II system was rinsed with air five times before a new sample was injected.

2.5.2. Apple flavor detection through GC-IMS

To identify the volatile compounds of apple samples, we incorporated a slightly modified static headspace gas chromatography-ion mobility method using a spectrometer (GC-IMS, G.A.S. FlavourSpec®; Dortmund, Germany) equipped with an MXT-WAX capillary column (30 m × 0.53 mm × 1 μm, Shimadzu, Tokyo, Japan) (Ge et al. 2020). Apple homogenates (2.00 ± 0.01 g) were placed in 20-mL headspace glass vials to ensure the accuracy of the analysis during the experiment and then incubated at 40 °C for 10 min with agitation at 500 rpm. And 500 μL of each sample was injected by CTC-PAL at 85 °C, separated by GC at 40 °C, and detected by positive ion mode of IMS cell at 45 °C with 3H ionization source (300 MBq activity) at 6.5 keV. Each spectrum was the average of six scans obtained by using an injection pulse width of 150 μs, and a repetition rate of 21 ms. Pure nitrogen was ramped up from 2 mL/min to 100 mL/min over 18 min, and finally maintained at this rate for 10 min, which was used as a carrier gas. The drift gas flow rate was 150 mL/min (nitrogen), and the drift tube length was 5 cm with a constant voltage of 400 V/cm at a temperature of 45 °C. N-alkanes (2 mL, 1 mg/L) was followed the same protocol as external standard.

2.5.3. Apple flavor detection via HS-SPME-GC-MS

The flavor detection via HS-SPME-GC-MS followed the method by Roberts,

Pollien, & Milo (2000). Apple slices (1.00 ± 0.01 g) were placed in 20 mL-headspace glass vials, and quantitatively analyzed for volatile compounds by GC with 10 μL DL-2-octanol as an internal standard (8.27 mg/kg). Samples were incubated at 50 ºC for 20 min and had flavor compounds extracted using 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB). The fiber was then desorbed at 200 ºC for 2 min at the inlet of the GC-MS (Shimadzu, QP 2010 Plus, Tokyo, Japan). The desorption product was injected using a split-less injector at 200 ºC with helium as a carrier gas at a constant flow rate of 1 mL/min. The product was separated using a DB-WAX (30 m × 0.25 mm × 0.25 μm, Agilent, California, USA) column. The oven temperature was programmed as follows: at first it was kept at 40 ºC for 3 min, then increased to 120 ºC at a rate of 5 ºC/min, immediately increased to 200 ºC at a rate of 10 ºC/min, and at last kept at 200 ºC for 5 min. A mass spectrometer was operated in the electron ionization mode with 70 eV. The ion source and transfer line temperatures were adjusted to 200 °C and 250 °C, respectively. The detector was set to scanning mode and varied from 35 to 500 m/z with a maximum scan speed of 2000 u/s. The scan time was initially at 0.1 s, and then ranged from 0.1 to 0.22 s when SIM mode was applied. A solvent delay of 1.5 min was applied to prevent damage to the filament in the ion source. Shimadzu software GC-MS solution was used to process the data automatically. Once the pure reference compound was unavailable, tentative identification based on characteristic fragments was used for quantification in the extensive screening of flavor compounds. Retention indices were used to identify the

potential flavor compounds based on gas chromatography mass spectrometry data.

2.6. Non-target metabolomics of apple

Cleaned control and CYF-treated apples were sampled for skin, pulp, and core, sliced, freeze-dried, and ground into powder, respectively, which was balanced mixed and dissolved in an extraction solution (70% methanol) for 12 h at 4 °C, with vigorous agitation three times. The mixture was then centrifuged for 10 min at 10000 *g* to obtain a supernatant. After filtration through a 0.2 μm filter, 2 μL of the supernatant was analyzed via UPLC-MS/MS (Waters MA, USA). The UPLC was equipped with a C18 column (Waters ACQUITY UPLC HSS T3, MA, USA, 1.8 μm, 2.1 mm × 100 mm) at 40 °C. The mobile phase comprised pure water: acetonitrile containing acetic acid (0.04%) in the following ratios: 95:5 (v/v) at 0 min, 5:95 at 11 min, 95:5 at 12.1 min, and 95:5 at 15.0 min. A triple quadrupole (QQQ) detector was used with an ESI temperature of 500 °C, a voltage of 5500 V, a curtain gas at 25 psi, and high collision-activated dissociation (CAD) (Chen et al. 2009). During detection, a sample for quality control (mixture of samples with equal proportions) was obtained and analyzed every 10 test samples to monitor the stability of the UPLC-MS/MS analytical system.

2.7. Transcriptomics of CYF-treated apples

Control and CYF treatment samples were frozen in liquid nitrogen and stored at -80 °C. Total RNA was extracted using the EasyPure Plant RNA Kit (TransGen Biotech, Beijing, China) according to manufacturer instructions and analyzed by

NanoDrop (Thermo Fisher Scientific, MA, USA) for quality and concentration. cDNA libraries were constructed according to Gasic, Hernandez, & Korban (2004), inspected with Agilent 2000 (Agilent, California, USA), quantified by quantitative real-time PCR (Q-RT-PCR, ABI-7500, Thermo Fisher Scientific, MA, USA), and sequenced with Illumina HiSeq (Metware, Wuhan, China).

2.8. Data analysis

Nutritional components were quantified relatively by integration of peak areas according to a calibration curve of corresponding standard solutions and analyzed with one-way ANOVA and Duncan's test at the 0.05 significance level using SPSS 18.0. In E-nose test, Alphasoft V12.44 was used to analyze the data. Principal component analysis (PCA) was used for visually assess similarities and differences between treatments and group the samples. Discriminant function analysis (DFA) was used to further minimize the within-group variance and maximize the between-group variance. In the GC-IMS test, Laboratory Analytical Viewer (LAV) software and GC × IMS Library Search were used to analyze the data. The volatile compounds were qualitatively analyzed with GC-IMS Library Search. The differences in volatile compound contents between the control and the CYF treatments were compared using Gallery Plot plugin in LAV. In the HS-SPME-GC-MS test, flavor composition was identified according to the NIST11 databases and relatively quantified by integration of peak area and the comparison with internal standard compound. In the non-target metabolomics analysis, metabolites were identified according to the Metware and

Metabolomics Databases and quantified by the multiple reaction monitoring (MRM) mode in QQQ with Analyst 1.6.3. The peak area of each detected compound was calculated and corrected by integration. MetaboAnalyst 3.0 was used to analyze the enrichment and metabolic pathways of the differential metabolites. In the transcriptomics test, the visualized data were transferred into raw reads by CASAVA and cleaned-up by eliminating reads with adapter sequences (over 10% unconfirmed base and more than 50% low quality base). The assembled contigs were compared to HISAT 2 and quantified by fragments per kilobase of transcript per million mapped reads (FPKM) (Kim, Langmead, & Salzberg 2015, Pertea et al. 2015). Differential expression genes were analyzed by DESeq2. The Gene Ontology Database was used to classify genes.

3 Results and Discussion

3.1. CYF deposition in apple

CYF deposition in fresh apples varied with application dosage. The initial deposition in apples were 25.03 ± 2.45 mg/kg in CYF_1.5 and 72.62 ± 2.23 mg/kg in CYF_5. The higher the CYF concentration applied to the apples, the greater the deposition in the same treatment. The spectrum of CYF is shown in Fig. S2. CYF detection was strictly performed according to the method of Guo et al. (2018), with a detection limit of 4.2-7.1 μg/kg and a quantification limit of 15 μg/kg.

3.2. Changes in the nutritional composition of CYF-treated apples

3.2.1. Changes in soluble sugar content

The total contents of sugars were not varied in CYF treatments compared with control (Fig. 1a, 185.32 ± 2.50, 179.12 ± 4.66, and 183.37 ± 2.04 mg/100g in CYF_CK, CYF_1.5, and CYF_5, respectively), while the constitutions of soluble sugar changed. In CYF treatments, contents of glucose and D-sorbitol were decreased (5.78 ± 0.06, 6.59 ± 0.03, 6.08 ± 0.04 mg/100 g for glucose, 2.58 ± 0.12, 2.32 ± 0.11, and 2.29 ± 0.09 mg/100 g for D-sorbitol in CYF_CK, CYF_1.5, and CYF_5, respectively) while contents of fructose and sucrose were increased (4.55 ± 0.01, 3.32 ±0.03, 2.91 ± 0.04 mg/100 g, 2.86 ± 0.06, 4.5 ± 0.12, 4.16 ± 0.12 mg /100 g for fructose and sucrose in CYF_CK, CYF_1.5, and CYF_5, respectively) in CYF treatments compared with control. These results indicated that the conversions between different kinds of soluble sugars were different in control and CYF-treated apple. However, there was no regular changes of the sugar compositions along with the CYF dosages.

Glucose and fructose are the two main reducing sugars in *Fuji* apples. They are major products of photosynthesis and are assimilated as sucrose and D-sorbitol. However, sucrose may be degraded into glucose and fructose by sucrase, while D-sorbitol may be metabolized into fructose and glucose by sorbitol dehydrogenase and sorbitol oxidase, respectively (Desnoues, Génard, & Quilot-Turion, 2018). Thus, soluble sugar metabolism might be modulated by CYF. D-Sorbitol and glucose were converted into fructose, while sucrose was inhibited to be hydrolyzed into fructose, leading to the accumulation of fructose in apples.

268 3.2.2. Changes in organic acid content

269 Malic and tartaric acids are the two main organic acids in apple. Malic acid
270 content increased in CYF treatments compared with the control (Fig. 1b, 34.98 ± 0.12,
271 38.96 ± 0.18, 37.22 ± 0.10 g/kg in CYF_CK, CYF_1.5, and CYF_5, respectively).
272 CYF_1.5 induced more accumulation of malic acid than CYF_5. Similar results were
273 observed in tartaric acid (5.51 ± 0.02, 9.79 ± 0.17, and 7.97 ±0.01 g/kg in CYF_CK,
274 CYF_1.5, and CYF_5, respectively) and lactic acid (2.01 ± 0.01, 3.16 ± 0.01, 2.34 ±
275 0.00 g/kg in CYF_CK, CYF_1.5, CYF_5, respectively), while the citric acid didn't
276 change in contents (1.59 ± 0.09, 1.79 ± 0.01, and 1.81 ± 0.01 g/kg in CYF_CK,
277 CYF_1.5, and CYF_5, respectively). These findings suggested that CYF modified the
278 organic acid composition in apples without a general concentration-dependent pattern.
279 The variations in total organic acid showed little difference between CYF_CK and
280 CYF treatments. However, the trend in variations was similar with that in malic acid,
281 tartaric acid and lactic acid, which might be due to other undetermined trace organic
282 acids. Considering the changes in malic acid, tartaric acid and lactic acid contents in
283 CYF treatments, CYF_1.5 seemed to induce greater changes than CYF_5. Organic
284 acids are important not only for fruit flavor but also for plant defense responses, and
285 their synthesis is reportedly promoted under minor external stimuli (Mithöfer &
286 Boland 2012). In this study, CYF_1.5 possibly acted as a minor external stimulus
287 inducing the production of defense compounds, such as organic acids, by apple. The
288 CYF_5 treatment might be too extreme and may have a negative effect on organic

289 acid production and accumulation.

290 3.2.3. Changes in amino acid content

291 Fifteen amino acids were detected in apple, including six essential amino acids,
292 namely Lys, Phe, Met, Ile, Leu, and Val (Fig. 1c). The abundance of amino acids
293 makes apple a highly nutritious fruit. In general, most amino acids varied in
294 concentration under CYF application, among which Asp (157.55 ± 2.21, 131.07 ±
295 0.87, and 110.37 ± 0.46 mg/100 g in CYF_CK, CYF_1.5, and CYF_5, respectively)
296 was the only one whose content was above the taste threshold (Schiffman, Sennewald,
297 & Gagnon, 1981). In the detected amino acids, Asp was accounting for the largest
298 proportion and variation, followed by Glu. Asp and Glu are the two main components
299 rendering a fresh and delicious taste to apples (Bachmanov et al. 2016). However, the
300 content of Asp was far more than the taste threshold while Glu was far less than the
301 taste threshold (Figure 1c), which made the Asp contributed largely to the taste
302 variation. Thus, the decreased level of Asp suggested that apple flavor might be
303 affected by CYF negatively. Amino acids, such as Gly, Ala, Ser, Thr, Pro, and His,
304 contribute partially to the sweet taste of apples (Bachmanov et al. 2016). The contents
305 of these amino acids in apples were increased after CYF treatments. However,
306 considering the high level of taste threshold of these amino acids, the sweet taste of
307 apple contributed by amino acids was less influenced by CYF. Similar results were
308 observed for amino acids contributing to fragrance and bitter taste. Overall, the
309 content of total amino acids was decreased by about 13.8% and 16.6% in CYF_1.5

and CYF_5 treatments, respectively. Thus, CYF treatment significantly varied the composition of amino acids, changed the nutritional composition, and altered the taste of apples. There was no apparent regularity in amino acid content variations, indicating a complex response of the apple to CYF application. Amino acids, especially those harboring side chains, such as Leu, are important precursors of some volatile compounds in fruits, promoting the production of 3-methylbutyl alcohol and its derivatives such as 3-methylbutanal and ethyl 3-methylbutanoate (Gonda et al. 2010). In addition, amino acids may be transformed into lipids and glucose through the tricarboxylic acid cycle (Hildebrandt, Nunes, Araújo, 2015). Thus, changes in amino acid content may further affect other metabolites in apples, thereby leading to changes in the nutritional quality and flavor of apple.

3.3. Changes in flavor composition

We used three different techniques to investigate the changes in apple flavor step by step. First, the difference between control and CYF-treated apples was distinguished by E-nose. Second, GC-IMS was used to identify trends in flavor composition changes. Finally, SPME-GC-MS was used to identify the formation of apple flavor and quantify flavor composition.

3.3.1. E-nose analysis

E-nose was used as an objective method to discriminate among apple flavors by transforming the chemical signals into electronic signals (Loutfi, Coradeschi, & Mani, 2015). The detected signals were first analyzed using PCA, which screened the factors

331 contributing most to the variation among the data. Thus, 113 detected signals were
332 converted into two principal components comprising a total of 98.04% in the CYF
333 treatments (Fig. 2a), with discrimination indexes of 83 and 89, respectively. These
334 two principal components reflected a difference in apple flavor composition between
335 control and CYF-treated apples. Flavor differences observed in CYF treatments,
336 showing that the application of CYF changed apple flavor composition, consistent
337 with changes observed in phytosiderophores in terbuthylazine-treated barley (Buono
338 et al. 2015).

339 Classification of samples with a minimum difference among replicates within
340 treatments was performed using DFA (Parsons & Jones 2000). The total discriminant
341 function of DF1 and DF2 was 100% in both CYF treatments (Fig. 2b). In addition, the
342 distance indexes between control and CYF treatments increased with CYF dose
343 (125291.7 for CYF_CK and CYF_1.5, 168890.0 for CYF_CK and CYF_5. Table S1),
344 which showed that flavor differences between control and CYF treatments increased
345 with CYF dose, thus suggesting that the CYF-induced changes in flavor composition
346 were concentration-dependent.

347 3.3.2. GC-IMS analysis

348 There were 37 well-known volatile compounds (including monomers and dimers)
349 identified, including 10 esters, 6 alcohols, 4 aldehydes, and 2 ketones (Table S2), all
350 of which conferred the fruity flavor and aroma on apples (Qi et al. 2020). The
351 variation of volatile compounds was analyzed by Gallery Plot. All detectable spectra

of apple flavor components were transformed into fingerprint, with points in the same line showing the detectable spectrum in one apple sample and points in the same row showing the same detectable compound in all detected samples (Fig. 3). Based on color intensity, the changes in the content of volatile components are summarized as follows. In CYF treatments, the contents of amyl acetate, ethyl propanoate, ethyl pentanoate, ethyl hexanoate, 1-propanol, and pentanal decreased with increased CYF dose. Meanwhile, ethyl acetate, amyl acetate, ethyl propanoate, ethyl isobutyrate, ethyl pentanoate, and ethyl hexanoate contents were higher in CYF_1.5 and lower in CYF_5 compared to the control treatment. The contents of 2-methylpropyl acetate, ethanol, and 1-propanol increased with increased CYF dose. Most of the components with varied contents were mainly responsible for apple flavor. These results suggest that CFY negatively affects the content of most volatile compounds under CYF treatments, especially esters, which are the main components of fruit aroma.

3.3.3. SPME-GC-MS analysis

There were 53 kinds of volatile compounds detected and identified, including 27 esters, 8 alcohols, 5 hydrocarbons, 2 ketones, 1 aldehyde, and 10 other kinds of compounds (Table S3). Dynamic principal component analysis (DPCA, Fig. S3) revealed differences between control and CYF treatments with respect to the identified compounds. Furthermore, flavor composition was relatively quantified by peak area in GC-MS. We found that apple volatiles mainly comprised esters, alcohols, and hydrocarbons (Table 1). In CYF treatments, the total ester and alcohol contents

373 decreased (84% in CYF_CK, 80% in CYF_1.5 and 71% in CYF_5), whereas
374 hydrocarbon content increased along with the CYF dose (13% in CYF_CK, 17% in
375 CYF_1.5 and 23% in CYF_5). The variations were disadvantageous to apple flavor,
376 consistent with results summarized above in 3.2.

377 Esters are the main flavor components in apple and are mainly synthesized by
378 esterification in amino acid and organic acid pathways (Pérez & Sanz 2008). Herein,
379 27 esters were detected, among which butyl acetate, 2-methyl butyl acetate, hexyl
380 acetate, hexyl butyrate, and hexyl 2-methyl butyrate possessed higher level of
381 contents than the odor threshold and contributed to the major components responsible
382 for apple fragrance (Colantonio et al. 2022). Butyl acetate, hexyl acetate, 2-methyl
383 butyl acetate, and hexyl 2-methyl butyrate, all with a relatively low odor threshold,
384 are responsible for the sweet fragrance in apples (Echeverría, Graell, & López, 2004,
385 van Gemert 2011, Niu et al. 2019). Contents of 9 kinds of ester above were increased
386 in CYF treatments compared with control. However, there were other 16 kinds of
387 detected ester, whose odor threshold were far lower than their detected contents, were
388 decreased in content. As shown in Table 1, total ester content decreased in CYF
389 treatments compared with the control, indicating that CYF had a negative effect on
390 apple flavor composition due to its influence on esters, especially on ethyl esters.
391 Meanwhile, hexyl butyrate and hexyl hexanoate, with high odor threshold, were less
392 significant to apple flavor formation, although their total content was also reduced in
393 CYF treatments compared with the control.

Alcohols are another main component of apple volatiles, exhibiting a gentle and fresh fruity flavor, originating from the reduction of amino acids, the oxidation of lipids, and carbohydrate metabolism (Kaever et al. 2014). Generally, saturated alcohol possesses a high odor threshold and contribute little to flavor formation, while unsaturated alcohol possesses a low odor threshold, whereby they overshadow the flavor of esters, making the fruit flavor more balanced (Jelen & Gacaka 2016). In this study, hexanol was the most abundant component in apple volatiles, furnishing fresh apples with a joyful aroma. However, hexanol decreased by about 3% in the CYF_1.5 treatment and by 20% in the CYF_5 treatment (3.69 mg/kg in CYF_CK, 3.59 mg/kg in CYF_1.5 and 2.22 mg/kg in CYF_5). In turn, octanol showed a relatively higher content (0.19 mg/kg in CYF_CK) than the odor threshold (0.054 mg/kg), the decrease of which would attenuate the aroma of fresh lemons and roses. Regarding other alcohols detected in apple, their contents were either under the odor threshold or not detectable in some samples, making aroma comparisons impossible.

Hydrocarbons constituted the second largest proportion of volatiles in the composition of apple flavor. However, all hydrocarbons possessed a high odor threshold and contributed little to apple flavor formation. In CYF treatments, total hydrocarbon content increased with no apparent variation in species. As for other components under study here, such as aldehydes and ketones, total content did not vary significantly between CYF and control treatments.

In addition, attention should be paid to two kinds of compounds. The first one

included butanol, 1-methylnaphthalene, and 1,3-dichlorobenzene, exhibiting unpleasant aroma, whose contents were lower than the odor threshold (No. 28, 40, and 46 in Table S3). The other compounds were ethyl 2-methyl butyrate, 2-methyl butyl acetate, hexyl acetate, hexyl 2-methyl butyrate and α-farnesene (No. 2, 4, 9, 16, and 39 in Table S3), whose contents were higher than the corresponding odor threshold and exhibited pleasant aroma. These two types of volatile components increased along with CYF dose, but how the observed changes in these two kinds of compounds affect apple flavor formation is not clear at present. Whether such changes lead to an enhancement of pleasant aroma or not. The relationship between changes in pleasant and unpleasant flavor components needs further investigation.

3.4. Correlations between nutrition and flavor composition

There is a number of datasets equal to 552. The relevant relationships between 23 nutritional compounds and 24 universal flavor compounds were analyzed and are shown in Fig. 4. Ethyl butyrate significantly correlated with Leu and Phe ($p < 0.01$) and with citric acid ($p < 0.05$). Butyl acetate positively and significantly correlated with Leu, Phe, and Pro levels ($p < 0.01$). In turn, butyl butyrate correlated positively with D-sorbitol and tyrosine. Meanwhile, hexyl acetate correlated positively with Asp, and 3-methyl-butyl 2-methyl-butyrate significantly and positively correlated with Phe, malic acid, glucose, and D-sorbitol ($p < 0.01$) but negatively with Ile, Leu, and lactic acid ($p < 0.01$). In turn, ethyl octanoate levels significantly and positively correlated with malic acid levels ($p < 0.01$), while isopentyl hexanoate significantly and

positively correlated with lactic acid ($p < 0.01$) but negatively with glucose ($p < 0.05$). Cis-3-Hexenyl valerate correlated positively with Asp ($p < 0.05$). Hexyl 2-butenoate and octanol significantly and positively correlated with malic acid levels ($p < 0.01$). Lastly, butyl heptanoate significantly and negatively correlated with Glu, Met, Pro, and tartaric acid ($p < 0.01$). Apple volatiles are derived from some primary and secondary compounds, by metabolic pathways, such as amino acid, organic acid, and glucose pathways (Siegmund, 2015). The correlation between nutrition and flavor compounds could explain the synthesis pathways of the flavor compounds. For example, Phe could be metabolized by a series of biochemical reactions into acetyl-CoA, which is the key metabolite that connects the metabolism of sugars, amino acids, and fatty acids. Thus, the correlation between Phe and ethyl acetate, butyl acetate and 3-methyl-butyl 2-methyl-butyrate could tell that there might be a metabolic pathway between Phe and ethyl acetate, butyl acetate and 3-methyl-butyl 2-methyl-butyrate. This was also fit for other nutrition and flavor compounds that were positively correlated. Additionally, volatile and nutritious compounds interact in a network, leading to a synergistic increase or decrease in levels in response to external stimuli, such as CYF.

3.5. Differential metabolites between control and CYF treatments

Retention times and ion intensities for quality control showed high repeatability, indicating that the analytical instrument, as well as the method, was steady and reproducible, resulting in reliable data (Fig. S5). When the CYF_1.5 treatment was

457 compared to CYF_CK, there were 39 differential metabolites (FC ≥ 2 or FC ≤ 0.5,
458 and VIP ≥ 1), among which, 35 were upregulated and four were downregulated.
459 Furthermore, there were 34 differential metabolites identified between CYF_CK and
460 CYF_5 treatments, 23 of which were upregulated whereas 11 were downregulated.
461 Among the identified and quantified compounds, there were 16 compounds
462 upregulated in both CYF_1.5 and CYF_5 treatments, and two compounds that were
463 downregulated (Table S4). All differential metabolites identified here were mostly
464 related to amino acids, polyphenols, organic acids, and lipids. In order to make a
465 comprehensive illustration on these differential metabolites and furtherly to unveil the
466 effects of CYF on apple metabolism, KEGG (Kyoto Encyclopedia of Genes and
467 Genomes) was conducted in this part. The differential metabolites were aligned into
468 KEGG database. Based on the molecular interactions in organisms, the aligned
469 metabolites in CYF_1.5 and CYF_5 treatments were enriched in 55 and 33 pathways
470 in the KEGG classification, respectively. The most susceptible metabolic pathways
471 were amino acids, polyphenols, organic acids, and lipid metabolism in both CYF_1.5
472 and CYF_5 treatments (Fig. S6).

473 All differential amino acids were upregulated in both the CYF_1.5 and CYF_5
474 treatments (Table S4). Generally, amino acids can be classified into four groups:
475 sweet (Gly, Ala, Ser, Thr, Pro, His), bitter (Val, Leu, Ile, Met, Trp, Arg), umami (Lys,
476 Glu, Asp), and fresh (Phe, Tyr, Cys) (Bachmanov et al. 2016). In addition, amino
477 acids are precursors of some flavor components, such as esters (Lara, Miró, & Fuentes,

2003). As shown in Table S4, all amino acids and derivatives in CYF_5 treatments (D-phenylalanine, L-alanine, L-tryptophan, L-proline, L-phenylalanine, L-histidine, L-carnosine, L-cystathionine, 3-(2-naphthyl)-D-alanine), except for L-cystathionine, were upregulated, and all, except for L-tryptophan were related to pleasant flavor (sweet and fresh). As a result, the amino acid components of pleasant flavor, or other flavor components related to amino acids, are seemingly promoted by CYF treatment. Catechins and their derivatives are polyphenols and amino acid precursors with aromatic groups, alkynes, and polyphenols (Saito et al. 2013). All catechins except one were upregulated under the CYF_1.5 treatment, which implied that the related pathways in apple were promoted by CYF application, possibly resulting in the synthesis of flavor components. Organic acids and lipids were upregulated and downregulated approximately by 50%, respectively. Considering that organic acids and lipids contribute little to flavor formation (Table 1, lipids were included in others), these two kinds of compounds might be involved in apple metabolism rather than directly contributing to flavor formation. However, no concentration-dependent behavior was observed according to compound-fold change in this case. Considering the results summarized in part 3.2 (Table S1, the increasing distance indexes in the three comparisons: CYF_1.5 & CYF_5, CYF_1.5 & CYF_CK, CYF_5 & CYF_CK, showing that CYF_5 changed more in flavor than CYF_1.5), CYF likely affects the intensity of changes in composition in a concentration-dependent manner, and our results allow us to conclude that CYF might have a concentration-dependent effect on

general apple flavor and affect apple metabolism through a complex and yet unknown mechanism.

3.6. Differential genes between control and CYF treatments

There were 1198 differentially expressed genes (DEGs) detected in CYF_1.5, compared to CYF_CK, with 612 upregulated and 586 downregulated genes (FC $\geq$ 2 or FC $\leq$ 0.5, and $p < 0.01$). Furthermore, there were 2144 DEGs detected in CYF_5 treatment, compared to CYF_CK, with 1707 upregulated and 437 downregulated genes. All DEGs were submitted to KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis and clustered into different pathways. The KEGG results simplified the thousands DEGs into tens pathways, which is easy to find how CYF affected the flavor and nutrition in gene level. The most notable pathway was the metabolic pathway in both CYF_1.5 and CYF_5 treatments (Fig. S8a and S8b). GO (Gene Ontology) analysis was also done in this part to describe the biological process (BP) that DEGs participated, the location of DEGs in cell (cellular component, CB), and the role that DGEs play (molecular function, MF). Fig. S8c and S8d show the top 20 statistics of GO enrichment in the CYF_1.5 and CYF_5 treatments, respectively. The most notable items in GO enrichment were those related to carbohydrate synthesis and cell metabolism. These results showed that CYF mainly affected primary metabolism in apple, which agreed with the results of the metabolomics analysis.

519 Furthermore, investigations were conducted into the general genes enriched in
520 carbohydrate synthesis and metabolic pathways. All genes were upregulated in both
521 CYF_1.5 and CYF_5 treatments. The carbohydrate biosynthetic process and
522 metabolism were the primary and central metabolic pathways in all types of features,
523 which related to amino acids, organic acids, and other metabolites through the
524 tricarboxylic acid cycle (Fig. 5). The effect of CYF on carbohydrate metabolism
525 might contribute to the synthesis of other small molecules, such as amino acids and
526 organic acids, leading to changes in apple flavor and nutrition. The pathways related
527 to carbohydrate synthesis and metabolism were more in the CYF_5 than those in
528 CYF_1.5 treatment, which could also be concluded from the comparison between
529 CYF_1.5 and CYF_5 treatments, showing a more severe impact of CYF at high
530 concentrations on apple metabolism, which might be the reason why CYF_5 led to
531 more severe flavor changes in apples.

532 **4 Conclusions**

533 In this research, we used multiple techniques to illustrate the variations in flavor
534 and nutrition of CYF treated apple. In conclusion, there were interconversions
535 between sugars and organic acids, respectively. The amino acid components
536 contributing to flavor and fresh taste (Asp) were negatively affected by CYF. The
537 changes in flavor components were strongly correlated with CYF dose, among which,
538 esters and alcohols, responsible for the pleasant fragrance of apples, were negatively
539 affected by CYF. In addition, nutritional and flavor compositions were closely

correlated, leading to a network and a complex response of apples to CYF application. Finally, our metabolomics study showed that CYF modulated basal metabolism in apples and further affected flavor components, among which amino acid, polyphenol, organic acid, and lipid metabolism were the most vulnerable to CYF. Transcriptomic studies suggested similar result that CYF promotes primary metabolism in apples, with upregulation of carbohydrate metabolism-, lipid-, and polyphenol synthesis-related genes. Overall, CYF promotes primary metabolism in apples, leading to significant changes in nutritional and flavor composition. Thus, the application of CYF should be strictly under the supervision and management to provide apple with better quality.

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References

Ayalew, Y., Retta, N., Desse, G., Mohammed, A. & Mellesse, A. (2017). Amino acid profile and protein quality in tuber and leaf of *Coccnia abyssinica* (Lam.) (Cogn.) accessions of Ethiopia. *Food Science & Nutrition*, *5*(3), 722-729. Doi: 10.1002/fsn3.452.

Bachmanov, A. A., Bosak, N. P., Glendinning, J. I., Inoue, M., Li, X., Manita, S., McCaughey, S. A., Murata, Y., Reed, D. R., Tordoff, M. G. & Beauchamp, G. K. (2016). Genetics of amino acid taste and appetite. *Advances in Nutrition*, *7*(4), 806-822. Doi: 10.3945/an.115.011270.

Brizzolara, S., Santucci, C., Tenori, L., Hertog, M., Nicolai, B., Stürz, S., Zanella, A. & Tonutti, P. (2017). A metabolomics approach to elucidate apple fruit responses to static and dynamic controlled atmosphere storage. *Postharvest Biology and Technology*, *127*, 76-87. Doi: 10.1016/j.postharvbio.2017.01.008

Buono, D. D., Astolfi, S., Mimmo, T., Bartucca, M. L., Celletti, S., Ciaffi, M. & Cesco, S. (2015). Effects of terbuthylazine on phytosiderophores release in iron deficient barley. *Environmental & Experimental Botany*, *116*, 32-38. Doi: 10.1016/j.envexpbot.2015.03.007

CAIA (2021). Report for Chinese apple industry development in 2020-China Apple Industry Association. (CAIA), C. A. I. A. Jinan Shandong.

Chen, Y. H., Zhang, R. P., Song, Y. M., He, J. M., Sun, J. H., An, Z. L., Dong, L. J., Zhan, Q. M. & Abliz, Z. (2009). RRLC-MS/MS-based metabonomics combined with in-depth analysis of metabolic correlation network: finding potential biomarkers for breast cancer. *Analyst*, *134*(10), 2003-2011. Doi: 10.1039/b907243h.

Colantonio, V., Ferrão, L. F. V., Tieman, D. M., Bliznyuk, N., Sims, C., Klee, H. J., Munoz, P. & Resende, M. F. R. (2022). Metabolomic selection for enhanced fruit flavor. *Proceedings of the National Academy of Sciences of the United States of America*, *119*(7), e2115865119. Doi:

10.1073/pnas.2115865119.

Desnoues, E., Génard, M., Quilot-Turion, B. & Baldazzi, V. (2018). A kinetic model of sugar metabolism in peach fruit reveals a functional hypothesis of a markedly low fructose-to-glucose ratio phenotype. *The Plant Journal*, *94*(4), 685-698. Doi: 10.1111/tpj.13890.

Domínguez, I., Ferreres, F., Pascual del Riquelme, F., Font, R. & Gil, M. I. (2012). Influence of preharvest application of fungicides on the postharvest quality of tomato (*Solanum lycopersicum* L.). *Postharvest Biology and Technology*, *72*, 1-10. Doi: 10.1016/j.postharvbio.2012.04.010.

Echeverría, G., Graell, J., López, M. L. & Lara, I. (2004). Volatile production, quality and aroma-related enzyme activities during maturation of 'Fuji' apples. *Postharvest Biology and Technology*, *31*(3), 217-227. Doi: 10.1016/j.postharvbio.2003.09.003.

Fellman, J., Miller, T. W., Mattinson, D. & Mattheis, J. P. (2000). Factors that influence biosynthesis of volatile flavor compounds in apple fruits. *HortScience*, *35*, 1026-1033. Doi: 10.21273/HORTSCI.35.6.1026.

Gasic, K., Hernandez, A. & Korban, S. S. (2004). RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. *Plant Molecular Biology Reporter*, *22*(4), 437-438. Doi: 10.1007/BF02772687.

Ge, S., Chen, Y., Ding, S., Zhou, H., Jiang, L., Yi, Y., Deng, F. & Wang, R. (2020). Changes in volatile flavor compounds of peppers during hot air drying process based on headspace-gas chromatography-ion mobility spectrometry (HS-GC-IMS). *Journal of the Science of Food and Agriculture*, *100*(7), 3087-3098. Doi: 10.1002/jsfa.10341.

Giaretta, A. G., Schulz, M., Silveira, T. T., Oliveira, M. V. D., Patrício, M. J., Gonzaga, L. V., Fett, R., Silva, E. L. D. & Wazlawik, E. (2019). Apple intake improves antioxidant parameters in hemodialysis patients without affecting serum potassium levels. *Nutrition Research*, *64*, 56-63. Doi: 10.1016/j.nutres.2018.12.012.

Gonda, I., Bar, E., Portnoy, V., Lev, S., Burger, J., Schaffer, A. A., Tadmor, Y., Gepstein, S., Giovannoni, J. J., Katzir, N. & Lewinsohn, E. (2010). Branched-chain and aromatic amino acid catabolism into aroma volatiles in *Cucumis melo* L. fruit. *Journal of Experimental Botany*, *61*(4), 1111-1123. Doi: 10.1093/jxb/erp390.

Gonzalez-Rodriguez, R. M., Noguerol-Pato, R., Gonzalez-Barreiro, C., Cancho-Grande, B. & Simal-Gandara, J. (2011). Application of new fungicides under good agricultural practices and their effects on the volatile profile of white wines. *Food Research International*, *44*(1), 397-403. Doi: 10.1016/j.foodres.2010.09.036.

Guo, J., Li, M. M., Liu, Y. G., Wang, F. Z., Kong, Z. Q., Sun, Y. F., Lu, J., Jin, N., Huang, Y. T., Liu, J. M., Francis, F. & Fan, B. (2018). Residue and dietary risk assessment of chiral cyflumetofen in apple. *Molecules*, *23*(5), 1060. Doi: 10.3390/molecules23051060.

Hildebrandt, T. M., Nunes N. A., Araújo, W. L. & Braun, H.-P. (2015). Amino acid catabolism in plants. *Molecular Plant*, *8*(11), 1563-1579. Doi: 10.1016/j.molp.2015.09.005.

Jelen, H. & Gacaka, A. (2016). Characterization of aroma compounds: structure, physico-chemical and sensory properties. In Guichard, E., Salles, C., Morzel, M. & Le Bon, A. M. (Eds), *Flavour*: 126-153.

Kaever, A., Landesfeind, M., Feussner, K., Morgenstern, B., Feussner, I. & Meinicke, P. (2014). Meta-

analysis of pathway enrichment: Combining independent and dependent omics data sets. *PLoS ONE*, *9*(2), e89297. Doi: 10.1371/journal.pone.0089297.

Kim, D., Langmead, B. & Salzberg, S. L. (2015). HISAT: a fast spliced aligner with low memory requirements. *Nature Methods*, *12*(4), 357-360. Doi: 10.1038/nmeth.3317.

Kong, Z. Q., Li, M. M., An, J. J., Chen, J. Y., Bao, Y. M., Francis, F. & Dai, X. F. (2016). The fungicide triadimefon affects beer flavor and composition by influencing *Saccharomyces cerevisiae* metabolism. *Scientific Reports*, *6*(1), 335-352. Doi: 10.1038/srep33552.

Lara, I., Miró, R. M., Fuentes, T., Sayez, G. & López, M. L. (2003). Biosynthesis of volatile aroma compounds in pear fruit stored under long-term controlled-atmosphere conditions. *Postharvest Biology and Technology*, *29*(1), 29-39. Doi: 10.1016/S0925-5214(02)00230-2.

Loutfi, A., Coradeschi, S., Mani, G. K., Shankar, P. & Rayappan, J. B. B. (2015). Electronic noses for food quality: A review. *Journal of Food Engineering*, *144*, 103-111. Doi: 10.1016/j.jfoodeng.2014.07.019

Mikulic-Petkovsek, M., Schmitzer, V., Slatnar, A., Stampar, F. & Veberic, R. (2012). Composition of sugars, organic acids, and total phenolics in 25 wild or cultivated berry species. *Journal of Food Science*, *77*(10), 1064-1070. Doi: 10.1111/j.1750-3841.2012.02896.x.

Mithöfer, A. & Boland, W. (2012). Plant defense against herbivores: Chemical aspects. *Annual Review of Plant Biology*, *63*(1), 431-450. Doi: 10.1146/annurev-arplant-042110-103854.

Niu, Y., Wang, R., Xiao, Z., Zhu, J., Sun, X. & Wang, P. (2019). Characterization of ester odorants of apple juice by gas chromatography-olfactometry, quantitative measurements, odour threshold, aroma intensity and electronic nose. *Food Research International*, *120*, 92-101. Doi: 10.1016/j.foodres.2019.01.064.

Nobuyoshi, T., Hirofumi, N., Yasuhiro, S. & Naoki, I. (2012). Development of a new acaricide, cyflumetofen. *Journal of Pesticide Science*, *37*(3), 263-264. Doi: 10.1584/jpestics.J12-03.

Osorio, S., Alba, R., Nikoloski, Z., Kochevenko, A., Fernie, A. R. & Giovannoni, J. J. (2012). Integrative comparative analyses of transcript and metabolite profiles from pepper and tomato ripening and development stages uncovers species-specific patterns of network regulatory behavior. *Plant Physiology*, *159*(4), 1713-1729. Doi: 10.1104/pp.112.199711.

Parsons, S. & Jones, G. (2000). Acoustic identification of twelve species of echolocating bat by discriminant function analysis and artificial neural networks. *Journal of Experimental Biology*, *203*(17), 2641-2656. Doi: 10.1242/jeb.203.17.2641.

Pérez, A. G. & Sanz, C. (2008). 4 - Formation of fruit flavour. In Brückner, B. & Wyllie, S. G. (Eds), *Fruit and Vegetable Flavour*, Woodhead Publishing**:** 41-70.

Pertea, M., Pertea, G. M., Antonescu, C. M., Chang, T. C., Mendell, J. T. & Salzberg, S. L. (2015). StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nature Biotechnology*, *33*(3), 290-295. Doi: 10.1038/nbt.3122.

Qi, W. Y., Wang, H. J., Zhou, Z., Yang, P., Wu, W. B., Li, Z. M. & Li, X. (2020). Ethylene emission as a potential indicator of Fuji apple flavor quality evaluation under low temperature. *Horticultural Plant Journal*, *6*(4), 231-239. Doi: 10.1016/j.hpj.2020.03.007.

Roberts, D. D., Pollien, P. & Milo, C. (2000). Solid-phase microextraction method development for headspace analysis of volatile flavor compounds. *Journal of Agricultural and Food Chemistry*, *48*(6), 2430-2437. Doi: 10.1021/jf991116l.

667 Saito, K., Yonekura-Sakakibara, K., Nakabayashi, R., Higashi, Y., Yamazaki, M., Tohge, T. & Fernie,
668 A. R. (2013). The flavonoid biosynthetic pathway in Arabidopsis: Structural and genetic
669 diversity. *Plant Physiology and Biochemistry*, *72*, 21-34. Doi:
670 doi.org/10.1016/j.plaphy.2013.02.001.
671 Schiffman, S., Sennewald, K. & Gagnon, J. (1981). Comparison of taste qualities and thresholds of D-
672 and L-amino acids. *Physiology & Behavior*, *27*, 51-59. Doi: 10.1016/0031-9384(81)90298-5.
673 Siegmund, B. (2015). 7 - Biogenesis of aroma compounds: flavour formation in fruits and vegetables.
674 In Parker, J. K., Elmore, J. S. & Methven, L. (Eds), *Flavour Development, Analysis and*
675 *Perception in Food and Beverages*, Woodhead Publishing**:** 127-149.
676 Śliwińska, M., Wiśniewska, P., Dymerski, T., Wardencki, W. & Namieśnik, J. (2016). Evaluation of
677 the suitability of electronic nose based on fast GC for distinguishing between the plum spirits
678 of different geographical origins. *European Food Research and Technology*, *242*(11), 1813-
679 1819. Doi: 10.1007/s00217-016-2680-6.
680 Tieman, D., Zhu, G., Resende, M. F., Jr., Lin, T., Nguyen, C., Bies, D., Rambla, J. L., Beltran, K. S.,
681 Taylor, M., Zhang, B., Ikeda, H., Liu, Z., Fisher, J., Zemach, I., Monforte, A., Zamir, D.,
682 Granell, A., Kirst, M., Huang, S. & Klee, H. (2017). A chemical genetic roadmap to improved
683 tomato flavor. *Science*, *355*(6323), 391-394. Doi: 10.1126/science.aal1556.
684 Uwe Meier, (2001). *Growth stages of mono- and dicotyledonous plants-BBCH Monograph (2nd Ed)*.
685 Federal Biological Research Centre for Agriculture and Forestry.
686 van Gemert, L. J. (2011). *Odour threshold-compilations of odour threshold values in air, water and*
687 *other media (2nd Ed)*. Oliemans Punter & Partners BV.

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689

690 **Table and Figure legends**

691 **Table 1.** Variations in apple volatiles compounds.

692 **Figure 1.** Content of nutritious components in apple from control and Cyflumetofen

693 (CYF) treatments (n= 3).

694 **Figure 2.** Flavor differences between apples in control and Cyflumetofen (CYF)

695 treatments.

696 **Figure 3.** Fingerprint of volatile compositions in control, CYF_1.5 and CYF_5

697 treatments.

698 **Figure 4.** Correlation between nutrition and flavor compounds.

699 **Figure 5.** Correlation of the amino acids and carbohydrates metabolism.

700

701

Table 1 Variations in apple volatiles compounds

Compounds	CYF_CK (%)	CYF_1.5 (%)	CYF_5 (%)
Esters	78	76	64
Alcohols	6	4	7
Hydrocarbons	13	17	23
Acids	0	0	0
Aldehyde	0	0	0
Ketones	0	0	0
Others	3	3	6

Note: CYF: Cyflumetofen; CYF_CK: apples treated with water; CYF_1.5: apples treated with 1.5 times of the Cyflumetofen (20%, SC) recommended dosage; CYF_5: apples treated with 5 times of the Cyflumetofen (20%, SC) recommended dosage. The percentage showed in this table were calculated from SPME-GC-MS data, which showed a general variation of the compounds of different categories.

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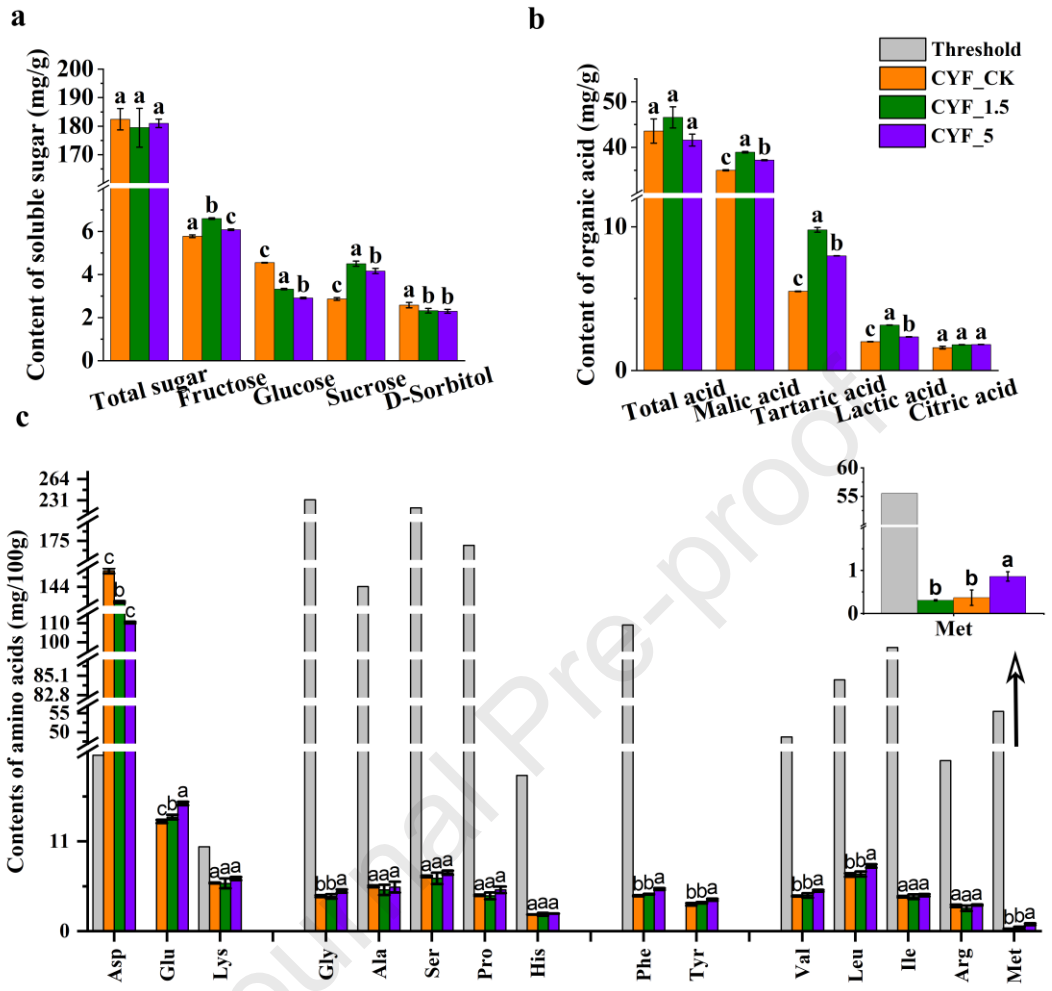


710

711 Figure 1 Content of nutritious components in apple from control and Cyflumetofen

712 (CYF) treatments (n= 3). (a): contents of soluble sugar; (b): contents of organic acid;

713 (c): contents of amino acids. There were 3 replicates and 4 apples per replicate in the

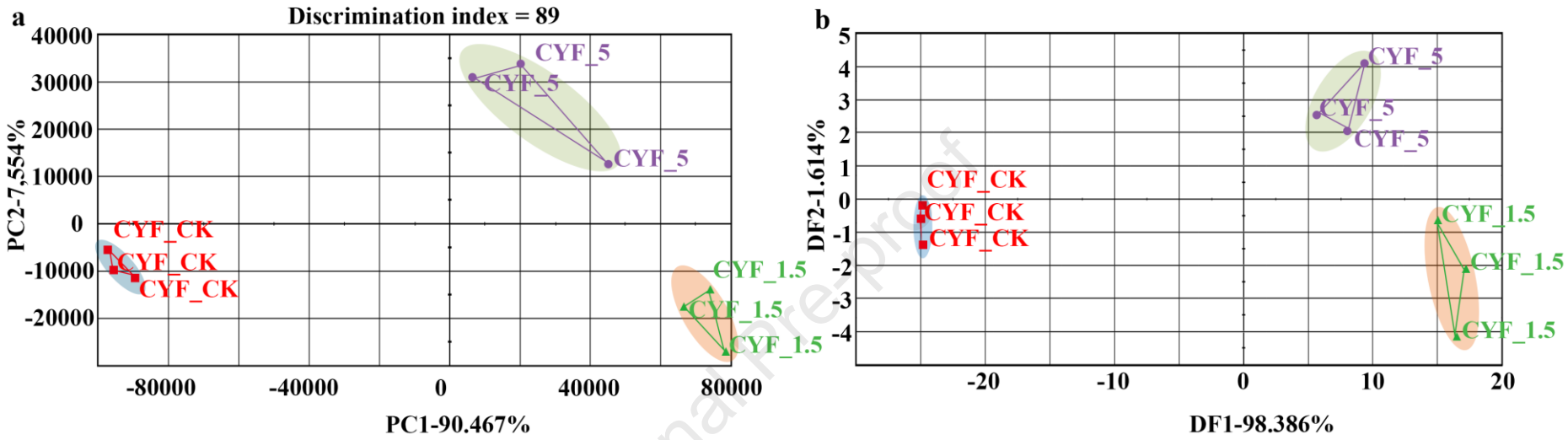
714 nutritious components test. The test was conducted twice. Data were shown as mean ±

715 standard deviation. The lowercases beyond the column suggested significant

716 difference between different treatments ($p < 0.05$). Values in (c) under every amino

717 acid under X axis are taste threshold referring to Schiffman et al. (1981).

718



719

720 Figure 2 Flavor differences between apples in control and Cyflumetofen (CYF) treatments. (a) Principal component analysis (PCA) result; (b)

721 Discriminant function analysis (DFA) results. There were 3 replicates and 4 apples per replicate in the E-nose test. The test was conducted twice.

722 Purple (with green background), green (with orange background) and red (with blue background) markers stand for CYF_CK, CYF_1.5 and

723 CYF_5 treatments, respectively.

724

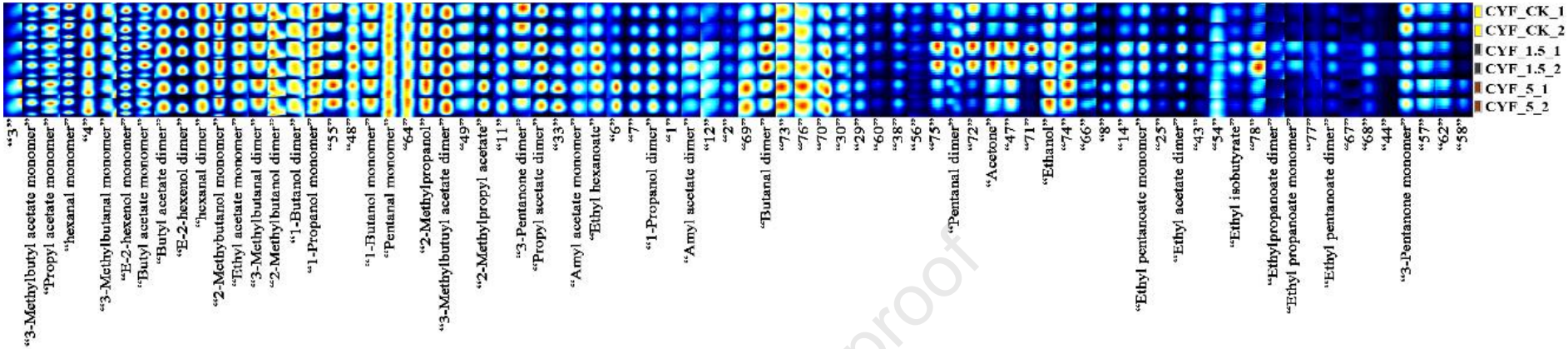
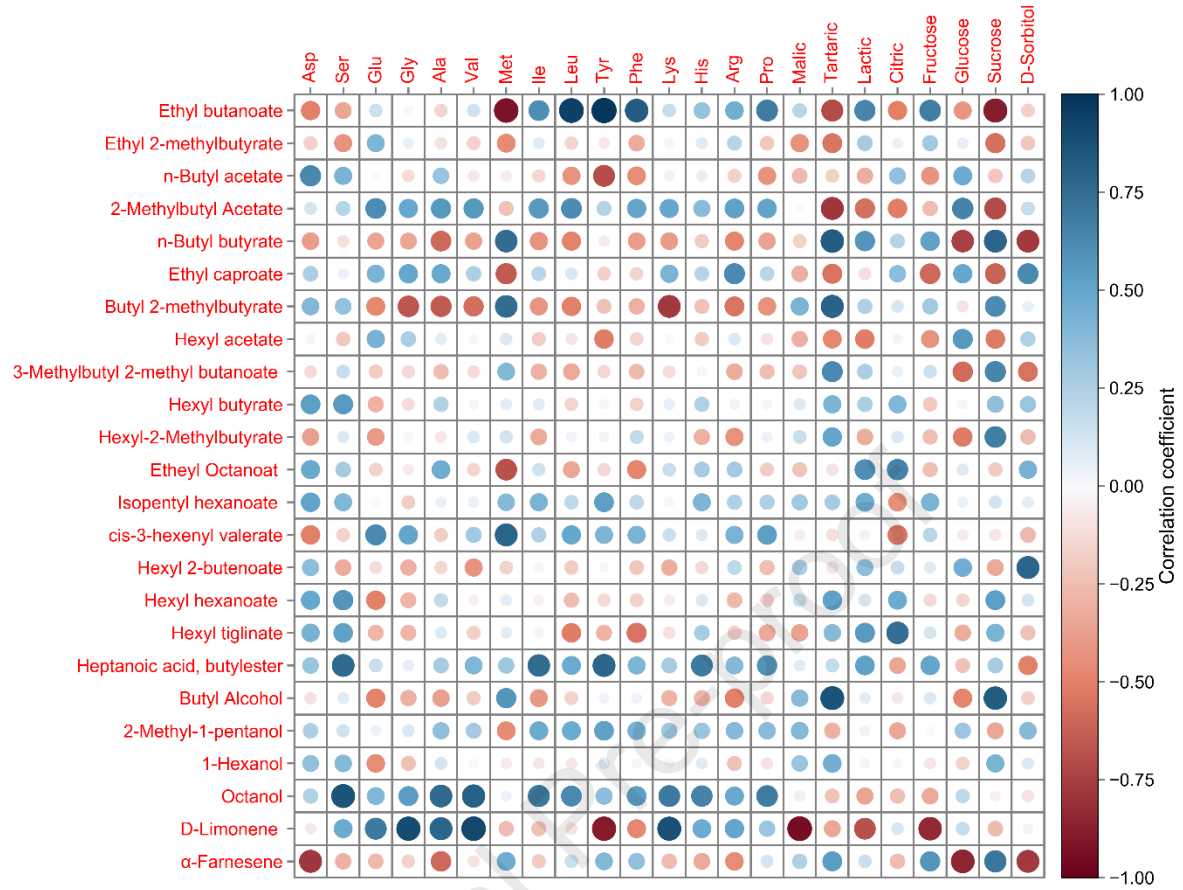


Figure 3 Fingerprint of volatile compositions in control, CYF_1.5 and CYF_5 treatments. There were 3 replicates and 4 apples per replicate in the GC-IMS test. The test was conducted twice. The color of each lattice showed the content of the compound. Dark red showed the highest content and dark blue showed the lowest content of the compounds.



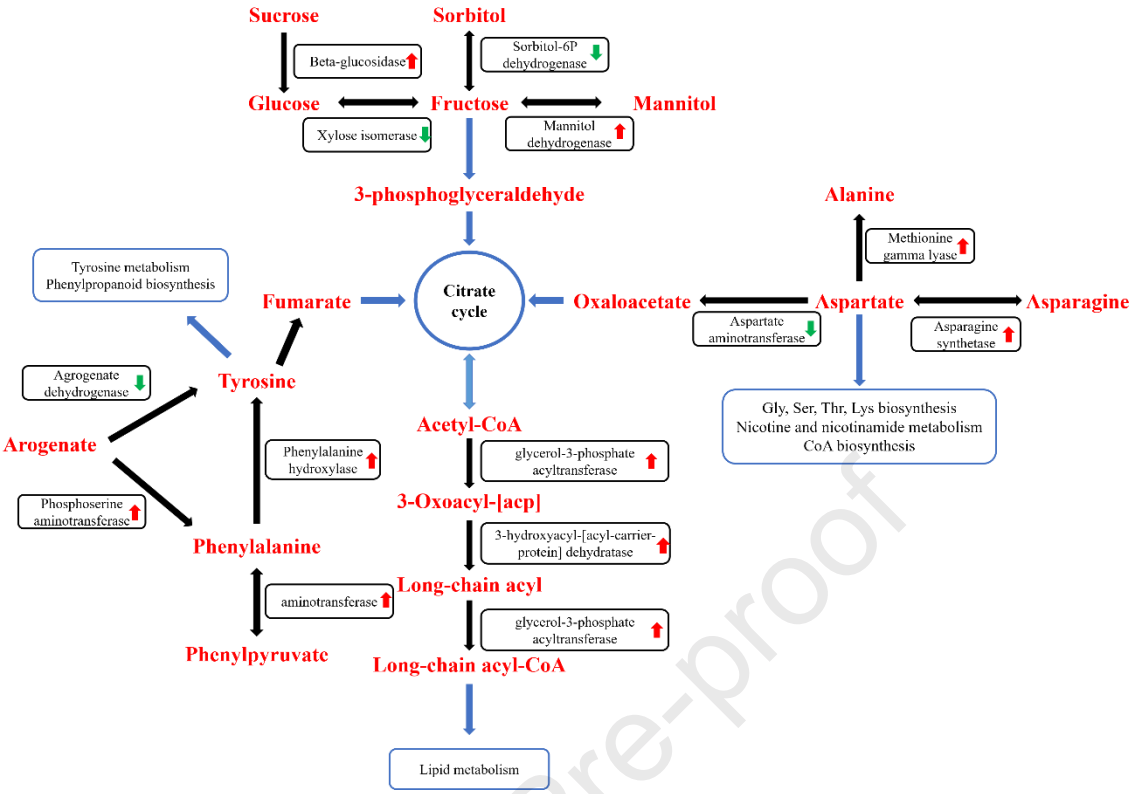
729

730 Figure 4 Correlation between nutrition and flavor compounds. The correlation was analyzed

731 with data from nutritious components test and flavor components test by HS-SPME-GC-MS.

732 There were totally about 48 apples involved in the correlation analysis from each treatment.

733



734

735 Figure 5 Correlation of the amino acids and carbohydrates metabolism. Red word showed the
736 key metabolites in the pathways. Black word in black frame showed the key genes functioned
737 in this pathway, with red arrow showing upregulation and green arrow showing
738 downregulation under CYF treatments. Black words in blue frame showed the downstream of
739 the metabolites.

740

741

Highlights

- Cyflumetofen changed the composition of soluble sugars and organic acids;
- Cyflumetofen decreased the content of amino acids related to palatability;
- Cyflumetofen decreased the content of esters and alcohols related to fragrance;
- Cyflumetofen altered amino acid-, organic acid-, polyphenol-, and lipid-metabolism related pathways in apple.

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: