RESEARCH ARTICLE



Metabolomics characterization of two saffron from Iran and China using GC–MS and LC–MS methods



Donglin Li^{1*}, Wenhui Zuo¹, Shuo Ma¹, Renjie Li² and Zhenfeng Ye²

Abstract

Saffron is a medicinally important species of the Iridaceae family. Saffron's quality depends on the concentration of secondary metabolites. In this study, GC–MS and LC–MS were utilized to dissect the different metabolites profile of two saffron produced in Iran and China. As a result, a total of 325 differential metabolites (237 from LC–MS, 88 from GC–MS) were identified. These differential metabolites were grouped into the following categories such as lipids, alkaloids, amino acids, terpenoids, organic acids, and flavonoids in saffron from the LC–MS analysis. The differential metabolites mainly concentrated on metabolic pathways, nucleotide metabolism, purine metabolism, and flavonoid biosynthesis. In Iran vs. China, the category of flavonoids includes Styrene, Apigenin-7-O-(6"-p-Coumaryl)glucoside, Houpulin F, Apigenin-5-O-glucoside, Tiglic acid; 2-Methyl-2-Butenoic Acid, p-Cymene, and 2,4-Nonadienal, (E,E)-. The terpenoids including downregulated Cafestol, rhodomollein XI, inumakiol G, D-Limonene, Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)- and upregulated rhodomollein XI were significantly different identified in Iran vs. China. In conclusion, the saffron from different cultivation areas possess many metabolites that are beneficial for health, including lipids, alkaloids, amino acids, terpenoids, organic acids, and flavonoids. Thus, compared with Iran, saffron from China have a higher medicinal value. This study provides a direction for the subsequent development and utilization of saffron.

Keywords Crocus sativus L., Metabolomics, LC-MS, GC-MS

Introduction

Crocus sativus L. (C. sativus), a perennial herb member of the Iridaceae family, is cultivated in various countries such as China and Iran (Pitsikas 2016). In China, *Crocus sativus L.* is known as "plant gold" and is a precious Chinese herbal medicine that has the functions of promoting blood circulation, resolving phlegm, generating new cells, relieving pain, and stimulating menstruation. Its metabolites of saffron can prevent lipid peroxidation and human platelet aggregation (Tabassum and Hamdani 2014). Chemical analysis of saffron indicates that the main compounds were water-soluble carotenoids (saffron), small amounts of monoterpene aldehydes (microtoxins and saffron), and flavonoids (quercetin and kaempferol), which have significant antioxidant and antitumor activity in cells (Lopresti and Drummond 2014). The quality of saffron is decided by its aroma, color, taste, as well as the concentration of its bioactive secondary metabolites (Cossignani et al. 2014). Previous studies have indicated that saffron and its active compounds possess antioxidant, antimicrobial (Wali et al. 2020), antiinflammatory, analgesic, antidepressant, anticoagulant (Khan et al. 2020), immunomodulatory (Yousefi et al. 2021), cytotoxic (Shakeri et al. 2020), and antitussive



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^{*}Correspondence:

Donglin Li

ldlcareer@126.com

¹ College of Urban and Rural Construction, Fuyang Vocational

and Technical College, 465 Funan Road, Fuyang City 236031, Anhui Province, China

² College of Horticulture, Anhui Agricultural University, Hefei 230036, China

(Saadat et al. 2018). However, the differential metabolites of saffron from different origins have been rarely studied.

The analysis of metabolic pathways in medicinal plants based on omics data has become one of the hotspots in variety selection at the molecular level. Metabolomics aims to comprehensively analyze all metabolites in biological samples and has great potential in elucidating plant metabolic processes (Jacobowitz and Weng 2020). For thousands of years, humans have relied on plant secondary metabolites as food, raw materials, and drugs. Many preparations used in traditional Chinese medicine are based on plant secondary metabolites to treat diseases. It has shown that more than 150 components existed in saffron stigmas (Samarghandian and Borji 2014). The active secondary metabolites produce an effect on the quality and efficacy of saffron (Carmona et al. 2006). The four main bioactive constitutes of stigma are crocetin, crocin, picrocrocin, and safranal (Moradzadeh et al. 2019). High-performance liquid chromatography-mass spectrometry (LC-MS) is a very useful tool widely used for the characterization of secondary metabolites in plants. Gas chromatography-mass spectrometry (GC-MS) provides supplementary data for LC-MS analysis, including small polar chemicals such as organic acids, sugars, amino acids, sugar alcohols, etc. (Scherling et al. 2010; Weckwerth 2010). Secondary metabolites have species specificity and typically have ecological functions. Therefore, the analytical instruments applicable to each category are different. Typically, primary metabolites are identified and quantified using gas chromatography/mass spectrometry (GC–MS), while secondary metabolites are identified and quantified using liquid chromatography/mass spectrometry (LC–MS) (Lee et al. 2013). Previous study has been combined with principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal PLS-DA (OPLS-DA) to explore different wines obtained from the same variety in different geographical regions (Papotti et al. 2013; Tan et al. 2020).

The quality of saffron mainly depends on the geographical region, which is not only related to changes in climate and soil conditions, but also to different harvesting times and post-harvest treatments (del Campo et al. 2010; Gresta et al. 2009). In recent years, in order to ensure the quality of saffron production in specific regions throughout its history, researchers have paid increasing attention. Due to the importance of ensuring the quality of saffron, which is crucial in international commercial agreements, an analytical method has been developed to better identify the compounds of interest, namely crocin, crocin esters, and crocin aldehyde (García-Rodríguez et al. 2014). Saffron's golden yellow-orange color is primarily the result of crocins, which are considered as major Page 2 of 9

constituents, whereas the picrocrocin and safranal are responsible for its bitterness and aroma, respectively. GC–MS and LC–MS were utilized to increase the number of detected metabolites, because each analysis platform can analyze different types of metabolites (Han et al. 2015; Wang et al. 2016). In this study, we applied GC–MS and LC–MS to profiling different metabolites in saffron flowers of Iran and China species. We evaluated the main components of saffron flowers from different origins can determine whether different cultivation techniques (such as irrigation, different contributions of nutrient solutions, and plant coverage) will affect the quality of saffron.

Materials and methods Plant materials

The experimental saffron seed ball "Yinghuai Elf" material comes from the Bingtao Farmers' Planting Cooperative in Qiaocheng District, Bozhou City, Anhui Province, China. The seed ball is healthy and free of pests and diseases, with an average single ball weight of 22.37 g. The saffron seed ball "Tarvand saffron" is made from highquality seed sources from Makyal Trading Company in Iran, with an average single ball weight of about 20.67 g. It is cultivated and propagated outdoors for many years. The "two-stage" cultivation method of indoor soilless cultivation and outdoor field breeding is adopted. The local altitude is 101 m, the average annual sunshine hours is 2184 h, the annual mean temperature is 10.6 °C and the average annual precipitation is 821 mm. The experimental plot was 150 m², with 3 replicates. Before planting, combined with fine preparation, 600 kg of fermented organic fertilizer and 15.00 kg of N-P₂O₅-K₂O (15-15-15) compound fertilizer were applied to each test site. Planting in the high beds, 12-20 cm in depth, 15×20 cm in the spacing between the plants and rows. The planting ball is placed flat with the top bud facing up. Saffron filaments were sampled at the flowering stage in November, and $N-P_2O_5-K_2O$ (15-15-15) fertilizer of 10 kg was applied in the middle of February of the next year combined with irrigation. Soil was kept moist and was loosened and weeded regularly, and watering was stopped when the stems and leaves on the ground turned yellow and withered in early May. Saffron seed bulbs were collected and treated and kept indoors for the dormant period.

Dry sample extraction

Using vacuum freeze-drying technology, the biological samples were placed in a lyophilizer (Scientz-100F); the samples were ground to powder by using a grinder (MM 400, Retsch). Next, 1200 μ L of pre-cooled 70% methanolic aqueous internal standard extract was added into 50 mg sample powder. After centrifugation (rotation speed 12,000 rpm, 3 min), the supernatant was aspirated,

and the sample was filtered through a microporous membrane (0.22 μ m pore size) and stored in the injection vial for UPLC-MS/MS analysis (Zelena et al. 2009).

Conditions to the analysis of UPLC and MS/MS

Metabolomics analysis was conducted by Wuhan Met-Ware Biotechnology Co., Ltd. (www.metware.cn) following their standard procedures. Ultra-performance liquid chromatography (UPLC) and tandem mass spectrometry (MS/MS) were used to perform the data acquisition. The liquid phase conditions were used as previous study (Bujak et al. 2015).

GC-MS analysis

The gas chromatography was performed on a DB-5MS capillary column (30 m \times 250 µm i.d., 0.25 µm film thickness, Agilent J & W Scientific, Folsom, CA, USA) and was used 1 mL/min of constant-flow helium to separate the derivatives, and 1 µL samples were injected through an automatic sampler at a split ratio of 1:10. The inlet temperature is 280 °C; the transmission line temperature and the ion source temperature are 320 °C and 230 °C respectively. The heating procedure takes 50 °C as the initial temperature, lasts for 0.5 min, rises to 320 °C at the rate of 15 °C/min, and stays at 320 °C for 9 min. The mass spectrum adopts full scanning method, scanning rate is 10 spec/s, electron energy is -70 V, solvent delay is 3 min (Fiehn et al. 2008).

The raw data were firstly converted to mzXML format by MSConvert in ProteoWizard software package (v3.0.8789) (Horai et al. 2010) and processed using XCMS (Sud et al. 2007) for feature detection, retention time correction and alignment. The Ropls (Boulesteix and Strimmer 2007) software was used for all multivariate data analyses and modelings. Pearson correlation analysis was used to calculate the correlation and p values between metabolites and microbiome in our previous study (Xu et al. 2023), and heatmap was utilized to show significant positive or negative correlations between metabolites and microbiome.

Results

Untargeted metabolomics analysis

The base peak chromatogram (BPC) of negative ion mode (ESI –) and positive ion mode (ESI +) in LC–MS metabolite profiles is shown in Fig. 1. A total of 2458 metabolites were detected based on UPLC-MS/MS and GC–MS. The total ion chromatograms (TIC) of one quality control sample (QC) showed the summed intensity of all ions in the mass spectrum at different time points. The PCA was used to check the quality of the data from the GC–MS and LC–MS analyses. Clear group separation between Iran and China samples could be observed in the PCA score plots (Fig. 2). In the GC–MS analysis, the PC1 and PC2 explained 58.27% and 21.02% of the total variance of all samples. In the LC–MS analysis, the first PC1 and



Fig. 1 Base peak intensity (BPI) and total ion chromatogram (TIC) between Iran and China. A BPI for the positive ions; B BPI for the negative ions; C TIC for the GC–MS



Fig. 2 Principal component analysis (PCA) score scatter plot. A positive ions; B negative ions; C GC-MS

PC2 explained 53.06% and 9.55% of the total variance of all samples.

Metabolic profiles analyzed by GC-MS and LC-MS

To discover variables that account for group separation, a supervised OPLS-DA was performed. The results of OPLS-DA are displayed in Fig. 3, showing an appreciable separation between Iran and China samples. The model statistics, R2X, R2Y, and Q2, indicate that the models were robust without statistical overfitting. Moreover, the combination of VIP > 1 and *P* value < 0.05 was applied to screen out the differential metabolites between differential metabolites (237 from LC–MS, 88 from GC–MS) were identified. The details of top20 differential metabolites from LC–MS and GC–MS are shown in Table 1.

A total of 325 metabolites were divided into 26 classes, including 13.22% phenolic acid, 11.15% flavonoids, 10.98% terpenoids, 8.79% amino acids and derivatives, 5.78% organic acids, 5.13% alkaloids, 4.6% heterocyclic compound, 3.66% nucleotides and derivatives and so on (Fig. 4). The accumulation pattern of metabolites between Iran and China samples could be visualized through a heatmap hierarchical cluster analysis (Fig. 5). Some metabolites in Iran group were upregulated, but downregulated in China group, suggesting that there were significantly different metabolic processes in the two different groups.

Metabolic pathway of differentially abundant metabolites

The KEGG pathway analysis of differentially abundant metabolites was performed by MetaboAnalyst 4.0 to identify the metabolic pathways between different fermentation



Fig. 3 Orthogonal projections to latent structures discriminant analysis (OPLS-DA) score scatter plot

Scores OPLS-DA Plot

Table 1 Identification of differentially abundant metabolites in LC–MS and GC–MS in Iran vs. China comparison

Platform	Name	Class I	Fold_Change	p-value	Regulation
	(3S,13Z,16Z)-3-amino-4-hydroxy-2-methyldocosa-13,16-dien- 5-one	Lipids	6.77E-02	5.22E-08	Down
	Cafestol	Terpenoids	1.68E-01	3.34E-07	Down
	4-Hydroxyquinoline	Alkaloids	4.87E-01	6.38E-07	Down
	N-carboxy-N-(2-oxo-2-phenylethyl)-L-alanine	Amino acids and derivatives	2.89E-01	1.22E-06	Down
	4-[(2 s,3r,4r)-4-(3,4-dimethoxyphenyl)-4-hydroxy-2,3-dimethylbu- tyl]-2-methoxyphenol	Lignans and Coumarins	4.05E-01	1.53E-06	Down
	3,4-Dihydro-4-(4'-hydroxyphenyl)-5,7-dihydroxycoumarin	Lignans and Coumarins	4.35E-01	1.97E-06	Down
	4-hydroxy-3(3-methylbut-3-en-2-yl) benzaldehyde	Others	4.55E-01	2.22E-06	Down
	linolenoylethanolamine	Lipids	3.87E-01	2.90E-06	Down
	rhodomollein XI	Terpenoids	3.26E+00	2.94E-06	Up
	Tababiphenyl C	Others	3.18E+00	3.11E-06	Down
UPLC-MS/MS	Tyr-Gly	Amino acids and derivatives	4.81E-01	3.19E-06	Down
	Luteolin-6-C-(2"-glucuronyl)glucoside	Flavonoids	5.72E-02	4.50E-06	Down
	Butylphthalide	Others	4.41E-01	5.41E-06	Down
	β-Nicotinamide mononucleotide	Nucleotides and derivatives	2.13E-01	6.07E-06	Down
	inumakiol G	Terpenoids	2.67E-01	6.81E-06	Down
	14,15-Dehydrocrepenynic acid	Lipids	4.94E-01	7.92E-06	Down
	DL-Glyceric Acid	Organic acids	3.75E-01	9.91E-06	Down
	Uridine 5'-diphosphate	Nucleotides and derivatives	2.69E+00	1.42E-05	Up
	2-Methoxy-6-undecyl-1,4-benzoquinone	Quinones	2.86E-01	1.42E-05	Down
	N-Benzoyl-(2R,3S)-3-phenylisoserine	Amino acids and derivatives	4.38E-01	1.48E-05	Down
	Valproic Acid	Acid	3.73E-01	3.07E-06	Down
	o-Xylene	Aromatics	3.81E-01	8.76E-05	Down
	Acetic acid, 2-phenylethyl ester	Ester	2.28E-01	1.58E-04	Down
	Pyrazine, 2-ethyl-3,5-dimethyl-	Heterocyclic compound	3.27E-01	1.83E-04	Down
	trans-Linalool oxide (furanoid)	Heterocyclic compound	2.93E+00	2.27E-04	Up
GC-MS	D-Limonene	Terpenoids	2.68E-01	2.91E-04	Down
	1H-Pyrazole-1-carboximidamide, 3,5-dimethyl-	Heterocyclic compound	2.47E+00	3.31E-04	Up
	2-Acetyl-3,4,5,6-tetrahydropyridine	Heterocyclic compound	4.12E-01	3.54E-04	Down
	cis-3-Hexenyl cis-3-hexenoate	Ester	3.55E-01	3.81E-04	Down
	3-Hexene, 1-(1-ethoxyethoxy)-, (Z)-	Aldehyde	4.25E-01	4.84E-04	Down
	2,4-Hexadienal, (E,E)-	Aldehyde	5.00E-01	6.02E-04	Down
	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	Terpenoids	7.97E-02	6.69E-04	Down
	p-Cymene	Aromatics	3.23E-04	7.40E-04	Down
	1H-Pyrazole	Heterocyclic compound	4.85E-01	7.42E-04	Down
	2-Nonenenitrile	Nitrogen compounds	2.76E-01	7.71E-04	Down
	Dodecane, 4,6-dimethyl-	Hydrocarbons	2.95E-01	7.94E-04	Down
	Pyrazine, 3,5-diethyl-2-methyl-	Heterocyclic compound	4.13E-01	9.12E-04	Down
	Benzene, 1-methoxy-4-propyl-	Aromatics	4.75E-01	9.30E-04	Down
	Cyclohexanone	Ketone	4.54E-01	1.13E-03	Down
	Acetic acid heptyl ester	Ester	421F-01	113E-03	Down

The upregulated sample was the metabolites that were increased in the China compared with Iran. The downregulated sample was the metabolites that were decreased in the China compared with Iran

stages. All the different metabolites were annotated to the biological pathways listed in the KEGG database (Fig. 6). We summarized these differentially enriched metabolic pathways into metabolic pathways, nucleotide metabolism, purine metabolism, and flavonoid biosynthesis.

The most significantly different metabolites in two Saffron The twenty compounds that were the most significantly

the twenty compounds that were the most significantly different (VIP>1, and top 20) in all comparisons were obtained (Fig. 7A). In the Iran vs. China group, the major differential categories were flavonoids, phenolic acids,



Fig. 4 The circular graph of metabolite classes. Each color represents a metabolite class, and the area of the color block indicates the proportion of that class



Fig. 5 Heat map plots of upregulated and downregulated metabolites

organic acids, and alkaloids. In Iran vs. China, the category of flavonoids includes Styrene, Apigenin-5-O-glucoside, Apigenin-7-O-(6"-p-Coumaryl)glucoside, Houpulin F, Tiglic acid; 2-Methyl-2-Butenoic Acid, p-Cymene, and 2,4-Nonadienal, (E,E)-. For the differential metabolites identified based on screening criteria and the sensory flavor features annotated in each difference comparison group, the top 10 sensory flavors with the highest number of annotations were selected for display in Fig. 7B.

Discussion

Crocus sativus L. (C. sativus), a perennial herb member of the Iridaceae family, is a precious Chinese herbal medicine that has the functions of promoting blood circulation, resolving phlegm, generating new cells, relieving pain, and stimulating menstruation. Crocus sativus contains more than 150 volatile and aroma-yielding compounds along with carotenoids including zeaxanthin, lycopene, and various α - and β -carotenes (Srivastava et al. 2010). More than 300 plant chemicals extracted from saffron stigma belong to different categories of secondary metabolites, such as carotenoids, terpenoids, flavonoids, and anthocyanins, which have enormous therapeutic applications (Khan et al. 2021). However, the research about the differences of saffron from different origins was rarely reported. In the present study, we applied the LC-MS and GC-MS analysis to explore the different metabolites between Iran and China. As a result,



Fig. 6 Summary of pathway analysis with MetaboAnalyst



Fig. 7 Differences in the contents of top 20 metabolites and metabolite flavor wheel between Iran and China comparison. Green indicates downregulated metabolites; red indicates upregulated metabolites

a total of 325 differential metabolites (237 from LC–MS, 88 from GC–MS) were identified. A total of 325 metabolites were divided into 26 classes, including 13.22% phenolic acid, 11.15% flavonoids, 10.98% terpenoids, 8.79%

amino acids and derivatives, 5.78% organic acids, 5.13% alkaloids, 4.6% heterocyclic compound, 3.66% nucleo-tides and derivatives.

We also found a wide range of lipids, alkaloids, amino acids, terpenoids, organic acids, and flavonoids in saffron from the LC–MS analysis. Differential metabolites that were found in Iran vs. China comparison were (3S,13Z,16Z)-3-amino-4-hydroxy-2-methyldocosa-13,16-dien-5-one, 14,15-Dehydrocrepenynic acid and linolenoylethanolamine, which were belonged to the category of lipids (Table 1). N-carboxy-N-(2-oxo-2-phenylethyl)-L-alanine, Tyr-Gly and N-Benzoyl-(2R,3S)-3-phenylisoserine were identified as the category of amino acids and derivatives. Some metabolites from the LC–MS analysis are still unidentified.

Flavonoid compounds have various biological activities, such as anti-ulcer, anti-inflammatory, antioxidant, cytotoxic and anti-tumor, anti-spastic, and antidepressant effects (Dai and Mumper 2010). They are important secondary metabolites in plants because they provide protection against insects, viruses, and bacteria (Tan et al. 2020). The 'Flavonoid biosynthesis' pathway is relatively conserved and the most sufficiently explored biosynthetic pathway for plant secondary metabolites (Schijlen et al. 2004). Our results showed that 'Flavonoid biosynthesis' pathway was significantly enriched in Iran vs. China comparison. The synthesis of flavonoid compounds originates from the phenylpropanoid metabolic pathway, where the intermediate phenylalanine is produced through the catalysis of phenylalanine lyase (PAL), cinnamate-4-hydroxylase (C4H), and 4-coumarin CoA ligase (4CL) to produce 4-coumarin CoA, followed by chalcone synthase (CHS), chalcone isomerase (CHI), flavone 3-hydroxylase (F3H), and flavonol synthase (FLS) in CHS, CHI, F3H, FLS, and anthocyanin synthase (ANS) (Fowler and Koffas 2009). The biosynthetic pathways of flavonoids are well studied in many plant species (Gao et al. 2020; Li et al. 2019; Wu et al. 2022). In the Iran vs. China group, the category of flavonoids includes downregulated Luteolin-6-C-(2"-glucuronyl)glucoside, which may be the reason for medicinal differences between Iran and China.

Extensive chemical analysis shows that saffron stigma contains over 300 volatile and non-volatile compounds, belonging to different categories of secondary metabolites, including terpenoids, flavonoids, carotenoids, and anthocyanins. Among them, carotenoids are the main component of saffron, mainly responsible for the red color and aroma of saffron (Chahine and Chahine 2020). Small amounts of other components, such as terpenoids, flavonoids, anthraquinones, and anthocyanins, can also be extracted from the stigma and other parts of saffron, mainly petals, pollen, and corms (Gresta et al. 2008). The terpenoids found in the stigma and petals of saffron, such as saffron, exhibit significant anti-tyrosinase activity and are also one of the Page 8 of 9

best recovered components. In our study, the terpenoids including downregulated Cafestol, inumakiol G, D-Limonene, Bicyclo[3.1.0]hexane, 4-methylene-1-(1methylethyl)- and upregulated rhodomollein XI were identified in Iran vs. China. Furthermore, Crocin 4 was downregulated in Iran vs. China. We indicated that these different compounds may be the main reasons, which leads to the quality differences and medicinal value between Iran and China.

Conclusions

A total of 325 differential metabolites (237 from LC-MS, 88 from GC-MS) were identified. In our study, some of the metabolites were annotated as lipids, alkaloids, amino acids, terpenoids, organic acids, and flavonoids. Furthermore, the terpenoids including downregulated Cafestol, rhodomollein XI, inumakiol G, D-Limonene, Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)- and upregulated rhodomollein XI were significantly different identified in Iran vs. China. Crocin 4 was downregulated in Iran vs. China. We indicated that these different compounds may be the main reasons, which leads to the quality differences and medicinal value between Iran and China. Furthermore, we summarized these differentially enriched metabolic pathways into metabolic pathways, nucleotide metabolism, purine metabolism, and flavonoid biosynthesis. Our results indicated that some specific metabolites in saffron obtained different origins. Thus, compared with Iran, saffron from China have a higher medicinal value. This study provides a direction for the subsequent development and utilization of saffron.

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None.

Author contributions

DL contributed to the conception of the study. DL and WZ performed the data analyses. SM and RL contributed significantly in writing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests

The authors declare that they have no conflict of interest.

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