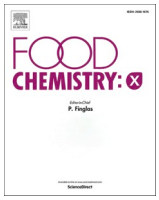
Food Chemistry: X 25 (2025) 102236

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Multi-component screening coupled with ultrasound-assisted green extraction based on HPLC-HRMS for bio-actives analysis in saffron (*Crocus sativus* L.)

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ARTICLE INFO

Keywords:
Crocus sativus L., bioactive components, HPLC-HRMS, multivariate statistical analysis, antioxidant

ABSTRACT

A database of 115 bio-actives from seven subclasses, including information on retention time, parent ion *m/z*, fragment m/z, and isotopic fit, was established, and an instrumental method for simultaneous analysis of these targets was optimised. An ultrasound-assisted preparation method at 50 °C for 50 min with a 1:60 solid-liquid ratio, water as the solvent was proposed for the screening of unknown components and the antioxidant analysis in saffron. The self-built database and untargeted analysis identified 32 and 103 bio-actives in saffron, respectively. The comparative analysis revealed that the antioxidant capacity of saffron petals was superior to that of stigmas. Correlation and multivariate statistical analyses indicated that terpenoids may be the main active substances in stigmas, while flavonoids and carboxylic acid derivatives play a pivotal role in conferring antioxidant activity to de-stigmatised saffron. The method serves as an ideal tool for mining the functional components of saffron and other agricultural products.

1. Introduction

Crocus sativus L., known as saffron, is a perennial bulbous herb of the genus Saffron stemless in the family Iridaceae. It is also referred to as "red gold" since it is one of the most expensive spices in the world. The stigma is proceeded to be a commercial for Chinese herbal medicine or industry. Previous studies have reported the medical properties of this ingredient, including anti-diabetic, pain-relief, anti-inflammatory, anti-tumour and anticarcinogenic effects (Chen et al., 2019; Hashemzaei et al., 2020; Mohammadi et al., 2021; Sun et al., 2020; Xu et al., 2022). Saffron stigma also contains a substantial number of important secondary metabolites, such as crocin, crocetin, safranal, and picrocrocin. These have been the subject of investigation with a view to establishing their capacity to exert a profound inhibitory effect on different cancer cell lines(Gezici, 2019; Liu et al., 2021). Moreover, the potential applications in retinal therapy(Heydari et al., 2023; Maccarone et al., 2016) and the improvement of metabolic processes in individuals with obesity (Abedimanesh et al., 2017) have been studied in saffron stigma. Saffron stigmas have thus been widely exploited as a high value-added nutrient product. Nevertheless, the fact that stigmas account for only about 0.3 % of the production of saffron petals and are not rationally utilised leads to the waste of plenty of saffron by-products (leaves, petals and bulbs) (Sánchez-Vioque et al., 2016). Those by-products are also potentially key sources of bioactive secondary metabolites (flavonoids, anthocyanins, etc.)(Belyagoubi et al., 2021).

In order to exploit the nutritional profile and key constituents of saffron, some analytical methods have been developed for the functional compounds with different structures, including spectrophotometry, nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC), and chromatography coupled with mass spectrometry, etc. Esmaeili, N et al. (Esmaeili et al., 2011)applied gas chromatography–mass spectrometry (GC–MS) for the analysis of 11 active compounds of saffron corms, where the samples were subjected to a series of procedures including extraction with methanol, refluxing in a water bath, and washing and derivatisation. Additionally, Natalia et al. developed and validated the high-performance liquid chromatography

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https://doi.org/10.1016/j.fochx.2025.102236
Received 11 December 2024; Received in revised form 22 January 2025; Accepted 24 January 2025
Available online 26 January 2025

with diode array detection (HPLC-DAD) method to determine saffron quality via a detailed assessment of its primary compounds(Moratalla-López et al., 2020). Furthermore, the metabolomics technique of liquid chromatography-mass spectrometry (LC-MS) was used to analyse the differences in the types and quantities of major metabolically active substances in saffron produced from two provinces by Wang et al. The results indicated that the types of the major active substances in saffron from different origins were largely the same despite their quantities (Wang et al., 2020). These methods still have the deficiencies of the single target, tedious pre-treatment preparation and poor qualitative accuracy, making it difficult to achieve rapid and efficient identification of the active compounds and micronutrient factors in saffron. However, high-resolution mass spectrometry (HRMS), as a tool of high-efficiency multi-component analysis, could not only perform with high throughput, high accuracy, and strong anti-interference ability but also achieve the retrospective analysis for the identification of unknowns and metabolomics, which compensates for the shortcomings of the current technology.

This study aims to establish a multi-component instrumental method and qualitative database by high performance liquid chromatography-quadrupole/Orbitrap high-resolution mass spectrometry (HPLC-Orbitrap-HRMS) to help investigate simultaneous and rapid screening techniques for bioactive components in saffron, which is beneficial to deeply excavate the active value of saffron by-products. With the proposed rapid, green and low-cost preparation procedure, the technique was applied to the targeted and untargeted screening of active components and antioxidant analyses in de-stigmatised saffron and saffron stigmas. Moreover, the correlations and differences of all the confirmed active ingredients in different saffron primary products were analysed by joint multivariate statistical analysis. The outcomes of this study provide a valuable reference for the in-depth development and utilisation of saffron by-products and nutritional mining of other agricultural products.

2. Materials and methods

2.1. Materials and chemicals

Fresh saffron was supplied from a cultivated base in Shanghai (31.23 N, 121.10 E). Saffron was cultivated with corms retained from previous years. Five hundred corms weighing 25–35 g without visible damage were chosen for the experiment (Zhou et al., 2022). Saffron corms were sown between November of the first year and April of the second year in a mixed matrix of 6:2:2 (*v*/v/v) peat, vermiculite, and perlite, fertilized with 30:10:10 (nitrogen, phosphorus, and potassium) every 20 days. The fresh stigmas and de-stigmatised portions of all the flowers were collected separately when they reached blossom, then transported to the laboratory at the Forestry and Pomology Research Institute, Shanghai Academy of Agricultural Sciences (31.22 N, 121.33 E) in an insulated box at 10 °C or less. Subsequently, fresh samples were conducted with two drying tests. One was direct drying to obtain yellow saffron petals (Direct) and red saffron stigma (saffron), and the other was multi-times drying with a small quantity each time to obtain purple saffron petals (Batch), which shows better sensory value due to the retained original purple colour. The dried samples were powdered after pre-cooling (−18 °C for 24 h) and vacuum-freeze drying and then kept at −18 °C before analysis. Three groups of saffron samples were used to statistical analysis with six duplicates.

Regents and solvents, including HPLC-grade methanol and acetonitrile, were purchased from Meker (Germany), whereas commercial standards of nutrients were obtained from Alta Technology Co., Ltd., Tianjin, China. 0.22 μm syringe needle filters: nylon (NySF) and nylon66 (Ny66) were provided by Jiangsu Lumeng Scientific Instrument Co., LTD (Jiangsu, China). Hydrophilic polytetrafluoroethylene (H-PTFE) and polyethersulfone (PES) were purchased from Shanghai Anpu Experimental Technology Co., LTD (Shanghai, China). In addition, the ABTS test kits, including 10 Mm Trolox solution, Enzyme solution, ABTS solution and dilution buffer, were available at Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Instruments and equipment

The liquid chromatography (Vanquish F) tandem Q/Orbitrap high-resolution mass spectrometer (LC- Q/Orbitrap-MSMS) system equipped with electrospray ionisation source and Xcalibur 1.2 data processing system (Thermo Fisher, USA) was applied for the acquisition and analysis. The following instruments were used as well in this study: the MX-F vortex mixer (Dragonlab, China), the 5415D centrifuge (Eppendorf, USA) and a heatable ultrasonic cleaner with 600 w power (KUDOS, Shanghai) for sample preparation, Milli-Q system (Millipore, USA) used for water purification, microplate reader purchased from Tecan (Männedorf, Switzerland) for the determination of total flavonoids and total polyphenols.

2.3. LC- Q/Orbitrap-MSMS method

The chromatographic separation was performed using a Syncronis C18 column (2.1 mm × 100 mm, 0.25 μm). 0.1 % formic acid in water and acetonitrile were used as the binary mobile phase. The gradient elution program was set as follows: from 0 to 1.5 min, 2 % B; 2 % B to 98 % B from 1.5 to 18 min; 100 % B during 18–23 min; 100 % B to 2 % B from 23 to 25 min; and 2 % B during 25–30 min. The injection volume was 5 μL, and the flow rate was 0.3 mL/min. The column thermostat temperature was set to 45 °C. The Q-Exactive mass spectrometer was operated in the heated electrospray ionisation mode (HESI). The MS parameters used for the full scan/data-dependent acquisition were stated as follows. The mass range of *m/z* was 100–1500; the curtain gas, spray gas, and auxiliary heated gas were 35 psi, 50 psi and 50 psi, respectively. The spray voltages for positive and negative full scans were 5000 v and 4800 v, respectively, and the corresponding capillary temperatures were 320 °C (+) and 300 °C (−). The default normalised collision energy (NCE) was gradient NCE: 20 eV, 40 eV, 60 eV. 30 and 10 arbitrary units (arb) were separately set for the sheath gas flow rate and auxiliary gas flow rate. The detailed information regarding mass spectral acquisitions of 115 active compounds is presented in Table S1.

2.4. Preparation of standard solutions

The stock solution for each compound with a concentration of 1000 μg/mL was prepared in a 10 mL volume flask by weighting 10 mg solid standard accurately and then dissolving in methanol. Ultrasonication was operated to prevent low solubility during the dissolving process. All stock solutions were subsequently stored at −20 °C, avoiding sunlight. The active compounds were classified into flavonoids, phenols, phenolic acids, alkaloids, polysaccharides and saponins, and others by the chemical structures. A mixed standard intermediate solution with a concentration of 10 mg/mL was prepared correspondingly for each category and was then stored at −20 °C away from sunlight with 3-month validity. The classification of 115 active compounds is shown in Table S1.

2.5. Pretreatments of samples

The 0.1 ± 0.005 g homogenised freeze-dried sample was added with 6 mL water, followed by being vortexed at 1600 g/min for 10 min, extracted ultrasonically at 50 °C ± 2 °C for 50 min, and then centrifuged at 5000*g*/min for 10 min after the extract cooling down to room temperature. This procedure was performed three times and the supernatants were then collected and filtered through 0.22 μm PES filters prior to the analysis of total phenols, total ketones, antioxidant capacity and HPLC-Q/Orbitrap-MS/MS.

2.6. Assay of total phenols and total flavonoids

The determination of total polyphenols (TPC) and total flavonoids (TFC) was referred to the method reported by Chu(Chu et al., 2018). The gallic acid was used as the standard based on the Folin-Ciocalteau method for TPC assay. The Folin-Ciocalteau reagent was diluted 10 times with distilled water, taking 25 μl of which to mix with sample extracts or different concentrations of GA standard solution in equal proportions. After a 5-min reaction in the 96-well plate at room temperature, 100 μl of distilled water and 25 μl of 20 % Na_2CO_3 solution were added in turn, mixing well and reacting for 30 min in the dark. The absorbance was measured at 760 nm wavelength with three duplicates. The TPC of the test samples was calculated according to the gallic acid calibration curve (10–200 ng/mL) and the results were expressed as gallic acid equivalent per gram of saffron (mg GA eq./g). Similarly, the TFC was assayed by the aluminium chloride colourimetric method with rutin as the standard. The reaction was carried out by aspirating 100 μl of 0.066 mol/L $NaNO_2$ solution into a 96-well plate, adding 50 μl of extract or standard solution respectively, mixing and reacting for 5 min at room temperature. Then, 15 μl of 10 % $AlCl_3$ solution was added, mixing and continuing the reaction for 6 min, followed by adding 100 μl of 1 mol/L NaOH solution to terminate the reaction. The absorbance was read at 510 nm wavelength with triplicates. The calibration curve of rutin (50–800 ng/mL) was used to calculate the TFC, and the results were expressed as mg of rutin equivalents /g of sample (mg RE eq./g).

2.7. Antioxidant activity

2.7.1. DPPH radical scavenging activity

The antioxidant activity of saffron (petals and stigma) was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay with reference to the previous study(Zhang et al., 2021). More specifically, 10 μL samples or the standard solutions were added with 240 μL of 0.1 mmol/L DPPH dissolved in ethanol and then incubated in the dark for 30 min. The UV absorbance was determined at 517 nm, and the results were expressed as Trolox equivalents. The percentage of DPPH radical scavenging activity was calculated as $[1-(A_0-A_1)/A_0] \times 100$ %, where A_0 and A_1 represent the absorbance of the control and the extracted sample, respectively.

2.7.2. ABTS radical scavenging activity

The ABTS free radical scavenging ability of saffron was assayed according to the kit instructions (Duan et al., 2025; Wang, Ma, et al., 2019). ABTS working solution was prepared from buffer I, ABTS solution II, and concentrated substrate solution III diluted 40 times with distilled water in the ratio of 76:5:4 according to the instruction manual. 10 μL of standard solution or test sample were taken to mix with 20 μL of enzyme solution IV diluted 10 times with buffer and then 170 μL of ABTS working solution were added, mixing well and reacting for 6 min at room temperature. The optical density values of each sample were measured at 425 nm wavelength by the enzyme-labeled instrument. The antioxidant capacity of the samples was expressed as Trolox-Equivalent Antioxidant Capacity (TEAC), using Trolox as the standard.

2.8. Data analysis

The self-built database with 115 active compounds was used to target identification. Non-target screening was performed by searching mzcloud, and online database from HMDB and PubChem (http://www.hmdb.ca/; https://pubchem.ncbi.nlm.nih.gov/). The peak intensities of components with target and non-target screening were loaded for preprocessing for missing value filling and normalization before multivariate statistical analysis. The orthogonal partial least squares discriminant analysis (OPLS-DA) was obtained with MetaboAnalyst 6.0 (https://www.metaboanalyst.ca), that enabled further data visualization and subsequent analysis. The three sets of data were modelled in two-by-two groups and the models were tested by a 7-fold cross-validation, and then the validity of the models was judged using the cross-validated R^2Y and Q^2. Based on the OPLS-DA analysis, orthogonal variables that were not correlated with the categorical variables were filtered out to obtain more reliable between-group differences in active ingredients(Chu et al., 2018). Other multivariate statistical analysis in terms of principal component analysis (PCA), cluster analysis, and heatmaps plotting and the correlation analysis between target results and antioxidant capacity were performed by R studio.

3. Results and discussion

3.1. Optimisation of instrument conditions

3.1.1. Chromatographic columns

Four columns with different packing materials, namely, ACQUITY UPLC C18 (2.1 mm × 100 mm, inner d = 1.7 μm), SB-C18 (3.0 × 50 mm, d = 2.7 μm), Syncronis C18 (2.1 mm × 100 mm, d = 2.1 μm) and EC-C18 (3.0 mm × 100 mm, d = 2.7 μm), were selected for the determination of a mixed standard solution with a concentration of 200 ng/mL. As shown in Fig. 1A, a maximum of 73 active components was detected by Syncronis C18, while 70, 65, and 64 targets were obtained by AC-C18, EC-C18, and SB-C18, respectively. in addition, according to the fold change method to calculate the fold change (FC) of the peak area of the active ingredients, there were 26 active ingredients with significant differences in peak shape and sensitivity (FC difference greater than 1). Notably, the peak areas of 12 compounds on the EC-C18 column were nearly two times higher than those on the other columns, which might be related to a better retention and elution ability caused by the longer column length and wider particle size of EC-C18, while the peak areas of 12 compounds on the fine particle size column were nearly two times higher compared to other columns, probably resulting from better separation by the smaller particle size, compared to wider particle size, especially for the compounds with very close chemical structures. However, there was a loss of information for a variety of targets between retention times (RT) of 5–12 min for EC-C18 and 7.5–14 min RT for SB-C18. Considering that Syncronis C18 has a certain compatibility in terms of particle size and column length, it was selected as the optimal column to capture as many molecular ion peaks and secondary information as possible.

3.1.2. Mobile phases

Three organic phases, including methanol with 0.1 % formic acid (FA-M), acetonitrile (ACN), and acetonitrile with 0.1 % formic acid (FA-ACN), and one inorganic phase of water with 0.1 % formic acid were chosen for the comparison in the detection of the standard solutions in order to get the optimal mobile phase formula. The same number of active compounds were detected, reaching 82, despite different organic phases being applied. However, significant differences have been presented in the FC values (FC > 1.5 or FC < 0.5) of the peak areas of 14 active compounds, including usnic acid, morin, inocembrin, etc. (Fig. 1B). According to the order of retention time, the moderate and less polar fractions inocembrin (RT11.81) and usnic acid (RT17.77) were more easily eluted by methanol and yielded higher responses. When applying 0.1 % formic acid in acetonitrile, the intensity of the compounds was not increased compared with acetonitrile elution. Therefore, acetonitrile and water with 0.1 % formic acid were selected as the mobile phases.

3.1.3. Mass spectrometry parameters

The inclusion list is one of the important ones for accelerating the acquisition of targeted and non-targeted information under data-dependent acquisition, and this procedure also improves the rate of secondary information acquisition in samples by the attention list. For the known list, this study optimised the collision energy in the DDA mode based on the characteristic of the measured secondary spectra, i.e., obtaining as much information as possible about fragments with

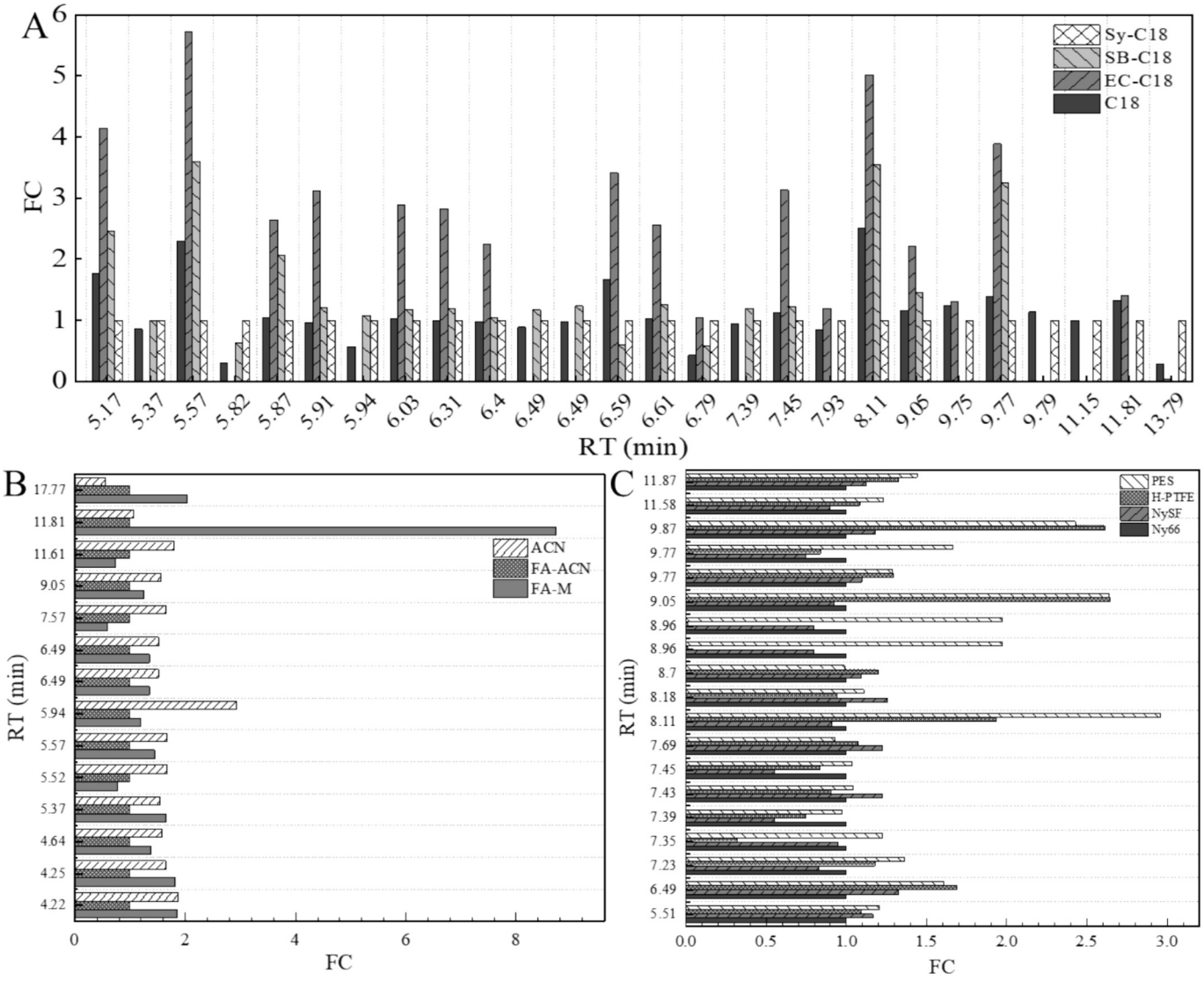

Fig. 1. FC values of the peak areas of the active compounds in different chromatographic columns(A), mobile phases(B) and filters(C).

abundance higher than the molecular ions *m/z* and with a mass number exceeding 100. At default gradient collision energies of 20, 40 and 60 eV, the single normalised collision energy(NCE) between 25 and 40 eV was used for apiin, ginsenoside Rc, myricitrin, and biochanin A, while 55–80 eV was implemented for twenty components, including vanillic, chrysin, some alkaloids, etc. The detailed optimal results were shown in Table S1. Moreover, unknown components outside of the list need to be considered, and if no further inclusion list entries are identified in a scan event, others m/z other than list entries will be selected and trigger collisions and fragmentation scans(Yang et al., 2023).

3.1.4. Needle filter

Membrane-sample compatibility is of importance in supporting efficient filtration and minimising resistance. Considering the wide polarity range of the compounds involved in this study, suitable filtration membranes can also reduce the loss of the targets during filtration. Four filters suitable for high aqueous phase (H-PTFE, NySF, Ny66, PES) were chosen for the comparison based on the result of the extraction solvent. The FC values with Nylon as the denominator are demonstrated in Fig. 1C under different filter membranes. It is shown that the properties of Ny66 and NySF are more similar due to the similarity in molecular structure. Isoferulic acid (RT 7.35) and luteolin (RT 8.96) were heavily adsorbed by the H-PTFE filter, resulting in lower peak areas. When using PES, the peak areas of six compounds were significantly higher than those in Ny66 and NySF, indicating that PES could obtain less loss of the targets in the solvent with a high proportion of aqueous phase. Thus, the PES filter was finally selected to filtration.

3.2. Optimisation of sample preparation

The active components in saffron have been reported including flavonoids, phenolic acids, polysaccharides, etc., which involve relevant extraction methods regarding traditional solvent extraction(Ramli et al., 2020), ultrasound-assisted extraction (UAE) (Xiaobin et al., 2018), microwave-assisted extraction (MAE) (Sobolev et al., 2014), supercritical fluid extraction (SFE) (Ahmadian-Kouchaksaraie & Niazmand, 2017) and pulsed electric field (PEF) (Liu et al., 2019), whereas UAE is still the prevailing method for component identification and isolation and purification due to its high operability, wide applicability and low cost. Moreover, the extraction of antioxidant constituents of saffron by UAE yielded extracts with higher purity in the research by Stelluti, S et al. (Stelluti et al., 2021). Thus, four UAE extraction parameters consisting of solid-liquid ratio, time (t), temperature (T) and solvent were then optimised with the performance indexes of TPC and TFC in the matrix of saffron petals to maximise the extraction of active compounds from the sample.

3.2.1. Optimisation of extraction solvents

Considering the polarity of the compounds in the matrix, the purpose of the extraction, the safety and the total cost, three solvents in regard to water, methanol, and ethanol were chosen to evaluate the effects on the extraction efficiency at the given extraction conditions (P = 100 W, solid: liquid = 1:40, t = 20 min, T = 35 °C). According to Fig. 2.A, water and methanol showed no significant differences between the extraction efficacy of polyphenols and flavonoid contents but were better than ethanol. It not only ensured the maximisation of extractions of active compounds but was more cost-effective and environmentally friendly. Moreover, the ultrasound-assisted extraction with safer solvents, namely water or a lower ratio of methanol, could achieve a similar extraction effect to that of the organic solvent impregnation method, as reported by Stelluti, S (Stelluti et al., 2021). Ömer Kayir's study also proposed that the highest antioxidant activity was obtained from the water extraction from the Crocus Ancyrensis (Kayir et al., 2023).

3.2.2. Optimisation of extraction time

The extraction efficiency of flavonoids and polyphenols was investigated at 20 min, 30 min, 40 min, 50 min and 60 min when being ultrasonically extracted (solid: liquid = 1:40, T = 35 °C) with water. The extraction time played an important role in extracting flavonoids and phenolic acid content of the saffron petals. As can be seen from Fig. 2B, with the increase of time, the total flavonoid content and total phenolic acid content in saffron petals fluctuate slightly, while too long time may lead to the destruction or degradation of the structure of flavonoids and phenolic acid (Ling et al., 2022). Therefore, extracting for 50 min could maximise the simultaneous extraction of flavonoids and phenolic acids with the content of 15.2 mg rutin (RE)/g and 14.6 mg gallic acid (GA)/g respectively.

3.2.3. Optimisation of extraction temperature

Under the given extraction condition (t = 50 min, solid: liquid = 1:40, water as the extraction solvent), five temperatures in terms of 25 °C, 35 °C, 50 °C, 60 °C, and 70 °C have been selected for the optimisation. The increase in extraction temperature facilitates the dissolution of flavonoid and phenolic acids due to intermolecular movement, but too high temperature will also cause damage to the structures of flavonoids and phenolic acid(Jha & Sit, 2022). It can be seen from Fig. 2C that the total flavonoid content in saffron petals increased first and then decreased, while the influence of temperature on total polyphenols was not significant. Taking the sum of TPC and TFC as the indicator, the sum of TPC and TFC with 32.6 mg/g was highest when the extraction temperature was 50 °C.

3.2.4. Solid-liquid ratio

The ratio of extraction solvent to substrate directly affects the efficiency of the substance's transmission. The effects exerted by different solid-liquid ratios (1:30、1:40、1:50、1:60、1:70, and 1:80) on the extraction efficiency of total flavonoids and total phenols were investigated. With the increase in solid-liquid ratios, the total flavonoid content in saffron petals showed a slight fluctuation, experiencing a decrease followed by an increase. When the ratio of solid to liquid increased from 1:30 to 1:60, the TPC increased continuously and tended to be stable

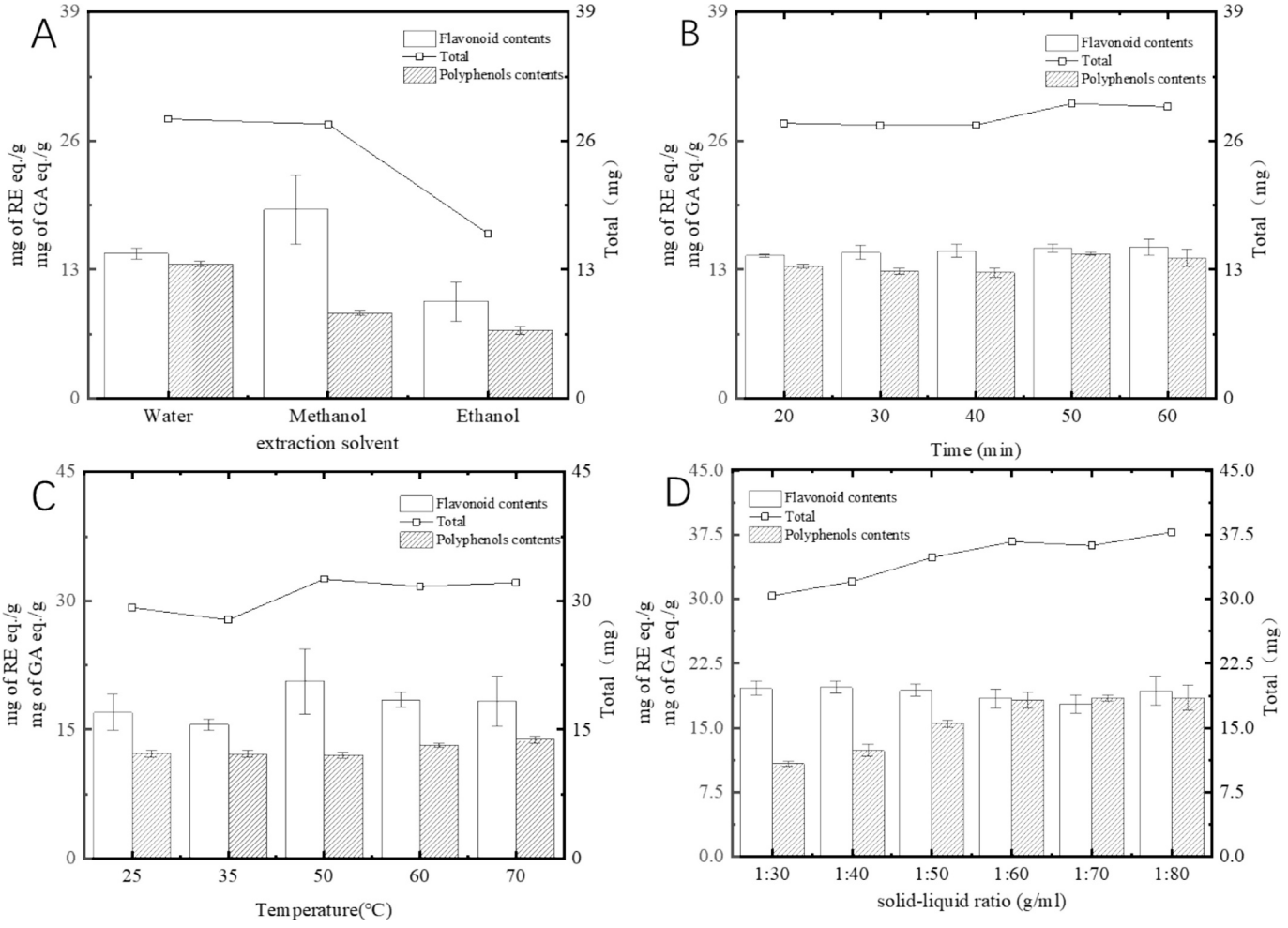

Fig. 2. Effect of different extraction conditions on the content of flavonoids and phenolic acids in extracted saffron (a) extraction solvent, (b) time, (c) temperature, (d) solid-liquid ratio.

after exceeding 60 mL/g (Fig. 2D). The highest total flavonoid and phenolic acid content was obtained at 1:60 solid-liquid ratio, which were 18.5 mg rutin (RE)/g and 18.2 mg gallic acid (GA)/g respectively.

3.2.5. The proposed preparation protocol

As shown in Fig. 3, the powdered saffron sample was treated with water and prepared with simple ultrasonic-assisted extraction, the extracts were used for component identification and antioxidant testing after centrifugation. Conventional maceration extraction using organic solvents (methanol or ethanol) has also been used for the extraction of natural components of saffron (Menghini et al., 2018), which is time-cost intensive, generally taking 24 h or overnight, and imposes a heavy environmental burden due to high solvent usage. In the study on the effect of stem age on the stigma activity of saffron (*Crocus sativus* L.), samples were macerated using 200 mL of methanol overnight (Razavi & Jafari, 2021). Novel extraction strategies including emulsion liquid membranes (ELMs), microwave-assisted extraction (MAE), and supercritical fluid extraction (SFE) have thus been developed and reported to improve the extraction efficiency and yield (Ali et al., 2022; Gupta et al., 2024), but they have focused more on the extraction of major biomolecules such as safranal and crocin. In the study conducted by Nescatelli et al.(Nescatelli et al., 2017), the saffron sample was subjected to MAE at a temperature of 40 °C for a duration of 5 h, when using a solvent mixture of water and ethanol in a 50:50 ratio. Safranal, picrocrocin and crocin-1 were extracted with a yield of 87 %, 80 %, and 68 %, respectively. Compared with these methods, the proposed preparation protocol in this study has the advantages of low energy consumption, low cost of reagents, ease of use, and applicability to a wide range of thermosensitive bioactive compounds. More importantly, according to the optimised operational variables confirmed in this study, water is used instead of hazardous organic solvents, which is considered more eco-friendly by improving safety and generating negligible environmental hazards and contaminants. Therefore, the adoption of this green extraction protocol (UAE at 50 °C for 50 min with a 1:60 solid-liquid ratio, water as the extraction solvent) could be of significance to the environmental benefits.

3.3. Screening and confirmation

The active compounds in saffron were extracted by optimal sample preparation, then analysed and scanned by UPLC-ESI-Q/Orbitrap-MS/MS to obtain full MS and MSMS data with positive and negative acquisition modes. The target screening database of 115 active compounds (Table 1) was used to rapid preliminary identification. The identification was carried out in accordance with the principles of compound confirmation and characterization of pesticides and veterinary drugs(Kong et al., 2018; Wang, Kong, et al., 2019): the deviation of the parent ion *m/z* less than 5×10^{-6}, retention time deviation of ±15 s, isotope mass deviation less than 5×10^{-6} and abundance deviation less than 20 %, and fragment ions satisfying at least one fragment with abundance greater than 1×10^{4} and deviation less than 1×10^{-5}. The results showed that 32 active ingredients were eventually identified by targeted screening in this study, including one amino acid derivative, 6 vitamins, 6 alkaloids, 19 flavonoids. The chromatograms of 32 active ingredients in the samples and standards were shown in Fig. S1. Twenty-seven and twenty-three ingredients, mainly flavonoids and vitamins, were identified in de-stigmatised saffron and stigmas, respectively, 18 of which were in common. Those found only in the stigma were mainly some alkaloids with trace amounts, but the variety is plentiful. The detailed information is shown in Table S2. Aside from defence against pathogens and herbivores, alkaloids also demonstrate health-promoting activities due to their potential to manage allergic conditions by alleviating inflammation and other allergy-inducing processes with their chemical structures, namely internal ring structure and functional groups(Javeed et al., 2024). In addition, alkaloids, especially pyridine alkaloids, are reported to exhibit therapeutic applications in central nervous system (CNS) diseases(Lin et al., 2020).

However, it is difficult to completely cover all active components in saffron by self-constructed database. Compared with the identification of hazardous substances such as pesticides and veterinary drugs, the identification of natural components has some limitations, such as uncertainty, unknown and accessibility of natural components, which will greatly increase the difficulty of database scaling. The dataset of tissue

Table 1
Comparation of Antioxidant activity of petals and stigmas.

	DPPH (umol Trolox/g)	$ABTS^{+}$ (umol Trolox/g)	FRAP (mmol Trolox/mL)
petals Batch	67.5 ± 6.37	95.18 ± 6.98	0.470 ± 0.062
petals Direct	54.3 ± 8.75	79.48 ± 10.1	0.505 ± 0.065
Saffron stigmas	9.49 ± 2.33	24.3 ± 4.60	0.330 ± 0.049

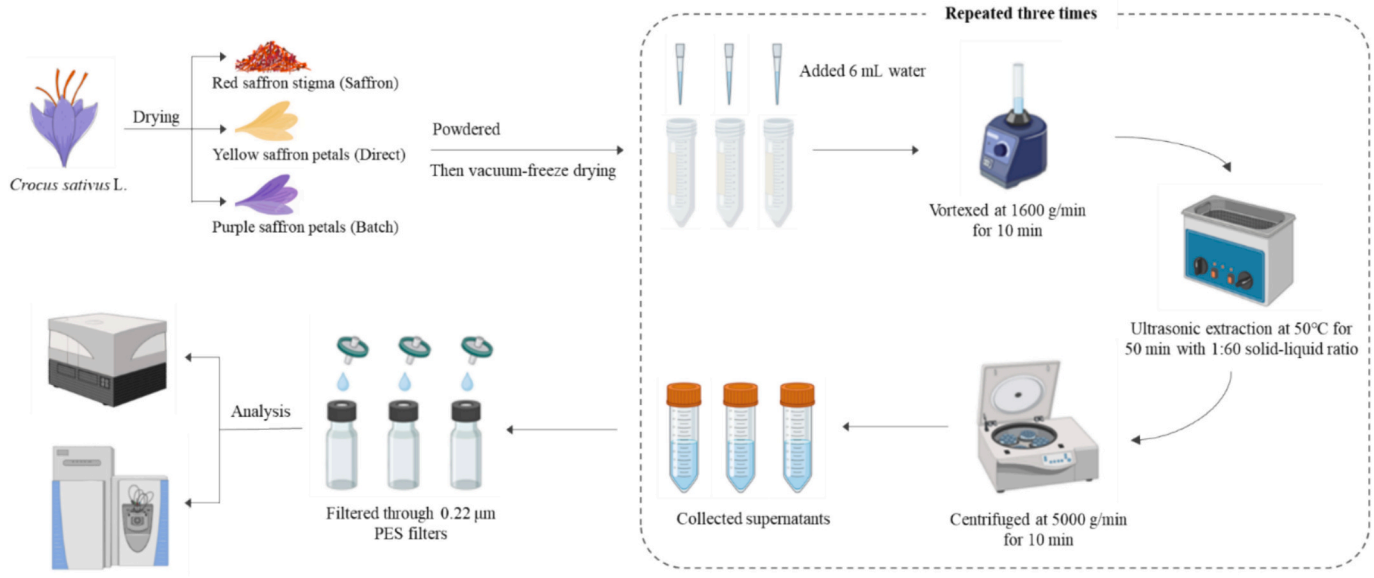

Fig. 3. The flowchart of the optimised extraction process.

samples collected by HRMS is itself highly complex and noisy, and non-targeted confirmation is commonly reported in metabolomics analyses, which is an important prerequisite for featured peak resolution (Samanipour et al., 2019). Consequently, this study incorporates non-targeted feature peak identification to compensate for the limitation of self-constructed databases. The online PubChem and Human Metabolomics Database (HMDB), as well as previous studies relevant to saffron (Choi et al., 2013; Li et al., 2015; Ni et al., 2022; Zhou et al., 2022) were used to non-targeted identification. Still, it has to be mentioned that the non-targeting confirmation of the bias in the identification of isomers of natural active compounds, if necessary, also requires the comparison of standard controls for confirmation in future studies.

The untargeted qualitative analysis of active compounds of saffron was performed by comparing the primary and secondary mass spectrometry features, and 103 compounds were confirmed by *m/z* of MS1 and MS2, mainly including amino acids and derivatives, carboxylic acids, fatty acids and derivatives, flavonoids and their glycosides, flavonols and their glycosides, terpenoids and other organic components as detailed in Table S2. Flavonoids, as one of the primary classes of secondary metabolites found in plant species, encompass flavonols and their corresponding glycosides and generally serve as a valuable source for food functionality, drug developments, pharmaceutical nutraceuticals, and cosmetic industries(Shen et al., 2022; Xing et al., 2021). Given that flavonoids are responsible for the colour and fragrance of the flowers, the wide range of solubility has been observed to meet various food processing needs, acting as pigments and flavour enhancers. In addition, the application of dietary supplements could be mainly attributed to the antioxidant potential induced by the free radical scavenging and metal-chelating properties of flavonoids. The antioxidative, antiallergic, and anti-inflammatory activities help in supporting health and preventing diseases like atherosclerosis, stroke, and neurodegeneration(Čižmárová et al., 2023; Kaushal et al., 2022). Moreover, the increasing trend for natural cosmetics products leads to a growing demand for naturally derived and biologically active ingredients. Flavonoids have thus gained increasing prominence from other botanical ingredients owing to the plethora of biological activities. Their anti-ageing, antiradical, anti-irritant, and anti-inflammatory properties result in the applications in preventing ultraviolet radiation and premature skin ageing, treating acne vulgaris and atopic dermatitis, and the cosmetology field(Čižmárová et al., 2023; Hou et al., 2019).

3.4. Antioxidant activity and correlations

In the study, two de-stigmatised saffron and saffron stigmas were used to evaluate the comparative antioxidant capacity. As shown in Table 1, the results of the antioxidant activity of each extract, the DPPH radical scavenging capacity of the sample extracts was observed in the following order from high to low: petals Batch (67.5 umol Trolox/g) > petals Direct (54.3 umol Trolox/g) > saffron stigmas (9.49 umol Trolox/g). Moreover, the $ABTS^{+}$ radical absorption capacity of the extracts was in the same sequence: saffron petals Batch (95.185 umol Trolox/g g) > saffron petals Direct (79.48 umol Trolox/g) > saffron stigmas (24.265 umol Trolox/g). The FRAP reducing capacity for petals B was 0.470 mmol Trolox/mL, 0.505 mmol Trolox/mL for petals D, 0.330 mmol Trolox/mL for stigmas. Thus, the antioxidant activity of de-stigmatised saffron dried with batches was slightly higher than that of direct drying. This is mainly due to the improvement in the process of the former, which allowed partial retention of anthocyanins in the leaves. However, it is clear from the corroborative results of the target screening that the flavonoids and phenolic compounds, the main contributors to antioxidant activity, did not undergo any significant degradation in these two drying processes. However, the antioxidant activity of the stigmas was much lower than that of the de-stigmatised saffron, which is consistent with previous findings(Kabiri et al., 2023).

The quantitative results of the targeted screening were plotted in correlation with the three indicators of antioxidant activity based on the screening and semi-quantitative analysis of the self-established database. As shown in Fig. 4A, most of the alkaloids and vitamins showed negative or no correlation with antioxidant activity, whereas pyridoxamine, nicotinic acid and most of flavonoids showed significant correlation with antioxidant capacity (Fig. 4B). Thus, flavonoids in de-stigmatised saffron are important contributors to antioxidant activity. A similar high correlation between flavonoid compound content and antioxidant activity is shown in grape pomace and rice with different colours in related studies(Peixoto et al., 2018; Shao et al., 2018).

3.5. Multivariate statistical analysis of saffron

The PCA plot of the active compounds of saffron stigmas and petals dried by two methods is shown in Fig. 5A. The proportion of the first principal component (PC1) and the second principal component (PC2) was 61.8 % and 19.2 %, respectively. The sum of PC1% and PC2% was greater than 80 %, indicating that multidimensional data has good reliability through unsupervised dimensionality reduction. Dots with different colours represent the samples from different subgroups. Circles are defined as 95 % confidence intervals (CI) and the samples show good intra-group reproducibility at 95 % CI. There is no crossover between the groups, which indicates a more significant difference between the groups. Additionally, the further the distance between groups represented, the greater the difference among the three groups of saffron. In the PC1 axis of the score plot (Fig. 5A), Batch and Direct groups of de-stigmatised saffron were positively distributed, and the stigma group showed predominantly negative distribution. In the PC2 axis, Batch and Direct groups appeared to be positively and negatively distributed, respectively, while stigma had a weak correlation with the PC2. According to the loading plot of PCA (Fig. 5B), where each point represents an active component, more than 90 active components fell in the positive axis of PC1, and these compounds were related to the de-stigmatised saffron samples. 38 components were negatively correlated with the variance contribution of PC1, and the majority of them originated from stigma. From this, we can conclude that the compounds that are far apart on the PC1 axis will be the key to the differences between the stigma and de-stigmatised saffron whereas the components further apart on the PC2 axis are crucial for the differences between the de-stigmatised saffron subjected to two drying methods.

Although PCA analysis could effectively reflect the main information, it is insensitive to the variables with low correlation. OPLS-DA, a multivariate statistical method with supervised pattern recognition, is beneficial to maximise group differences and discover the potential differential active compounds. In order to distinguish the differential metabolites, OPLS-DA model has been implemented to compare the components of de-stigmatised saffron dried by Direct and Batch, and two parts of saffron (Direct and Saffron stigma). According to OPLS-DA model of permutation test($n = 1000$), drying methods was verified by permutation test to obtain predictive ability ($R^2 = 0.992$) and interpretation rate ($Q2 = 0.976$), respectively. Similarly, the model of two parts of saffron was verified with R^2 (0.997) and Q^2 (0.995). It indicated that OPLS-DA demonstrated good model fitness and predictability, yielding large differences between groups. It also provided the baseline for subsequent screening for differential active components.

Variable Importance in Projection (VIP) between different models was determined based on OPLS-DA analysis, which measures the contribution of each variable to the classification of the model. Usually, the higher the value of VIP, the more important the component is in distinguishing different groups. The screening of differential fractions resulted in 65 differential active components under different drying methods, and 98 variable ones between the stigma and de-stigma parts of saffron based on VIP ≥ 1, indicating that the variation of active components in saffron was significant under different models. The differentials in drying modes and different parts of saffron are shown in Figs. 4B (VIP > 1.101) and 4C (VIP > 1.128). In Fig. 4B, 28 compounds were up-regulated and 24 significantly down-regulated by multiple-

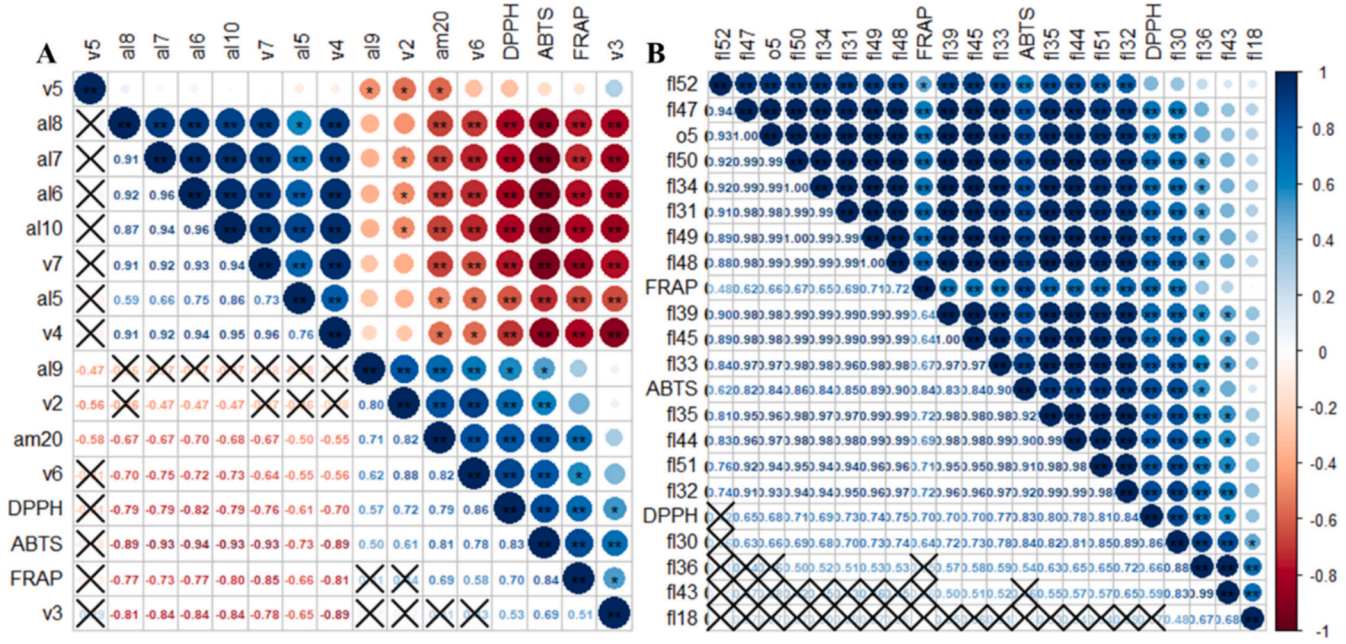

Fig. 4. Bubble plot of correlation between target identification components and antioxidant activity.

times drying in small batches (Batch), and when VIP $\geq$ 1, significantly up-regulated 27 constituents in direct drying, and this differentiation will be observed to be more remarkable compared to the 38 up-regulated in Batch. As shown in Fig. 4C, 39 active components were significantly up-regulated in de-stigmatised saffron and 11 fractions in stigma. They were mainly pharmacologically active terpenoids and other organic compounds such as (all-E)-crocetin, safranal, nepetaside, geranic acid, and picrocrocin. Thus, de-stigmatised saffron has a wide variety of components with antioxidant, antimicrobial and other activities besides the high value-added pharmacological active components, which also provide a basis for the deep processing value of saffron by-products.

All confirmed active components were normalised and plotted with cluster heat maps to visualise the pattern of differences more generally (Fig. 7). The saffron samples had good clustering properties within groups, while inter-groups of all observed indicators were grouped into four large clusters, echoing the OPLS-DA and PCA results. Compared with the two groups of de-stigmatised saffron, the relative concentrations (RCs) of 32 active compounds in saffron stigmas were higher, containing 10 terpenoids, 5 alkaloids, 4 organic compounds, 2 saccharides and 2 vitamins, 3 amino acids and fatty acids and others, which collectively determines the efficacy of stigmas in improving blood lipid metabolism, anti-ageing, anti-fatigue and etc.(Ghaffari & Roshanravan, 2019; Qadir et al., 2024). Regarding the colour characteristics of the thermograms, there were 27 compounds with higher RCs in the direct group compared to the batch group, while 32 active components with significantly higher RCs were in the batch group. It indicates that the former of these compounds are susceptible to the effect of processing time during drying, while the latter is sensitive to the temperature of drying. In addition, 37 active ingredients showed lower variance in the direct and batch group, demonstrating their better processing stability. In general, compared with direct drying, batch drying not only retained richer active ingredient information but also partially preserved the anthocyanin undertones of saffron petals, which resulted in better organoleptic qualities and improved consumer acceptance of saffron by-products. However, considering the findings of total antioxidant capacity, direct drying is a more reasonable approach given its cost-effectiveness and maximised retention of active ingredients when de-stigmatised saffron is utilised for active ingredient extraction and further processing.

4. Conclusions

In this study, a database of 115 active compounds containing chromatographic and high-resolution mass spectrometric information and an HPLC-Q/Orbitrap-MSMS method for simultaneous multi-component screening were developed. The database information was established based on the optimised acquisition and analytical parameters of standard solutions using HPLC Q-Exactive Orbitrap MS. A green, rapid, accurate, and cost-effective extraction protocol (UAE at 50 °C for 50 min with a 1:60 solid-liquid ratio, water as the extraction solvent) was proposed for ultrasound-assisted extraction of the active components in saffron. The pretreatment technique would decrease the energy input and organic solvent usage with negligible impacts on environmental hazards while facilitating extraction quality and yield, exhibiting eco-friendly benefits and safety improvements. Combined with HPLC-Q/Orbitrap-MSMS for data-dependent acquisition and rapid elucidation of the four dimensions of the database, the high-throughput identification and semi-quantitative analysis of different target components in saffron was achieved by the self-built database.

The method was applied to study the identification of active components and antioxidant activity of saffron, coupled with non-targeted confirmation of the unknown active components in saffron. 32 and 103 active substances were therefore identified by targeted and non-targeted analysis, respectively. Different parts of saffron, namely stigmas and de-stigmatised saffron, under two drying models (direct and batch drying), were assessed with comprehensive differential analysis (PCA, OPLS-DA and heatmap analysis) to obtain detailed annotations of the active components. The major pharmacologically active fractions were terpenoids, alkaloids and other organic compounds in saffron stigma, while flavonoids, amino acids and their derivatives and carboxylic acid derivatives were the predominant fractions in destigmatised saffron. It also further corroborated that $ABTS^{+}$ radical absorbing capacity, DPPH radical scavenging and reducing capacities of the active components in destigmatised saffron were higher than those in stigma. Based on statistical analysis, the internal differences of the active components in different drying methods were further explained, thirty-eight compounds were significantly up-regulated in batch-drying de-stigmatised saffron, suggesting that this drying method reduces the loss of active ingredient species and retains the more acceptable organoleptic qualities of the by-products of processing. Therefore, the results of this

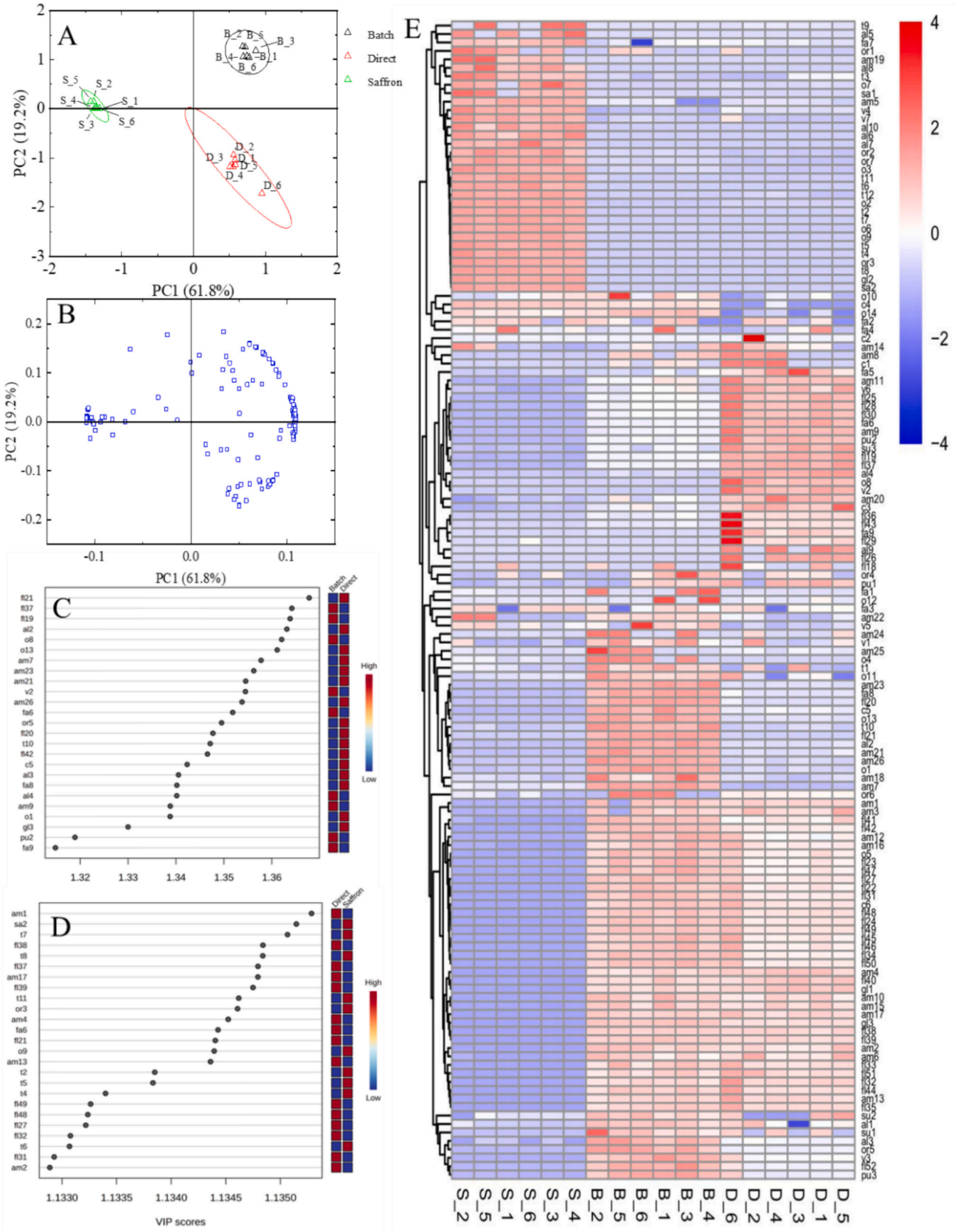

Fig. 5. The PCA score plot (A) and loading plot (B), VIP scores in OPLS-DA analysis (drying method(C), two parts(D)) and heatmap (E) of saffron.

study provide new insights into the extraction and isolation of active components in saffron and point to the further direction for the rational utilisation and development of saffron by-products. In addition, given the rising health consciousness, there is a growing demand for naturally derived and biologically active substances around the world. Saffron-derived bioactive compounds, especially flavonoids, could provide promising possibilities for further applications in food-processing, pharmaceutical, nutraceutical, and cosmetic industries due to their antioxidant, anti-inflammatory, and antiallergic properties. This technology can also be used as an efficient means to evaluate and analyse the effective active ingredients for the deep processing of saffron by-products, and provide reliable technical support for exploring and mining the characteristic nutritional factors of other agricultural products and edible plants.

Funding information

This work was supported by Shanghai Central Guided Local Science and Technology Development Fund Program (YDZX20223100003004), Shanghai Plant Seedling Histoculture Professional and Technical Service Platform (21DZ2292300).

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used DeepL Write in order to improve language and readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

CRediT authorship contribution statement

Shouying Wang: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Data curation. **Yiqing Song:** Writing – review & editing, Validation, Investigation, Formal analysis. **Miaomiao Chen:** Writing – original draft, Methodology, Investigation, Data curation. **Bing Bai:** Writing – review & editing, Investigation. **Lin Zhou:** Resources, Methodology, Formal analysis. **Changyan Zhou:** Formal analysis, Data curation. **Yongchun Zhang:** Validation, Funding acquisition. **Zhiying Huang:** Visualization, Supervision, Conceptualization. **Wenshuai Si:** Writing – review & editing, Project administration, Methodology.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.102236.

Data availability

Data will be made available on request.

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