P67: COMPARATIVE METABOLOMICS OF SAFFRON FROM DIFFERENT COUNTRIES, FARMING METHODS AND FOOD/DRUG CLASSES

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Abstract – Saffron is an invaluable medicinal crop. Saffron-specific phytochemicals have been well studied, yet the whole compositions and their variations remain to be clear. We analyzed six saffron products from different countries, farming methods and food/drug classes through metabolomics with a computational analysis system (Categorical Mapper). Total 5310 compositions were found, of which 3153 were identified, including 545 lipids and 257 drugs. High precision mass spectrometry and accurate identification are essential for assessment of food safety and medical applicability.

Keywords: categorical mapper, composition analysis, metabolomics, informatics, Saffron

1. INTRODUCTION

Saffron, which is the stigmas of *Crocus satisvus* L., is one of the most valuable medicinal crops and widely cultivated in the world. It is used as dye, food coloring, flavoring agent or herbal medicine (sedative, pain reliever or emmenagogue) [1-4]. Saffron-specific compositions and their regional and temporal differences have been reported [5-8], a whole spectrum of compositions and their variations remain to be clear.

Metabolomics that systematically identifies numerous compositions in one object is promising for composition analysis of natural products, but its primary output is primitive and dimensionally large. To make it more informative and understandable, we have developed a computational analysis system (Categorical Mapper) for metabolomics of natural products. The system identifies, classifies and visualizes compositions based on their chemical, biological and medical properties retrieved from public databases. Herein, using this system, compositions of Saffron from different countries, farming methods and food/drug classes are analyzed.

2. EXPERIMENTAL

2.1 Samples. We analyzed six commercial products of saffron as shown in Table 1. Note that each 3-letter sample ID denotes the producing area (country), farming method and food/drug class in the order. IOF was labelled as the organic product. Those unlabeled were presumed to be the products from conventional outdoor farming, while JCF is indoor-cultivated. CCD was sold as a crude drug and the rest as foods.

Table 1. Saffron samples used in this study.

ID	*Producing area*	*Farming method*		*Food/Drug class*
JCF	Oita, Japan	Conventional	indoor	Food
GCF	Greece	Conventional	outdoor	Food
SCF	Spain	Conventional	outdoor	Food
ICF	Iran	Conventional	outdoor	Food
IOF	Iran	Organic	oudoor	Food
CCD	Tibet, China	Conventional	outdoor	Drug

2.2 Extract Preparation. Each sample was ground to powder under liquid nitrogen. In a 2mL Eppendorf tube, 200mg of Saffron powder, 1.5mL of 80% methanol and one 5mm zirconia bead (#Z250-0001, BMS) were mixed and shaken up on QIAGEN Tissue Lyzer at speed 25 per second for 2 minutes, sonicated for 2 minutes and centrifuged at 15,000*g* for 10 minutes. The supernatant was collected, filtered first through 13mm GD/X syringe filter (#6874-130, Whatman) pre-equilibrated with 80% methanol, and then through Monospin C18 (#5010-21701, GL Sciences).

2.3 Mass Spectrometry. The extract was subjected to the LC-LTQ-Orbitrap Mass Spectrometer (ThermoFisher) in the positive ion mode, which was made accessible through a metabolite analysis service (Kazusa DNA Research Institute, Japan). Peaks of exact mass <1500 were traced.

2.4 Identification, Classification and Visualization of Compositions. By the computational analysis system (Categorical Mapper), all chemical, biological and medical information for small compounds covered by metabolomics were retrieved from public databases

(KEGG, LIPIDMAPS, HMDB, ChEBI and PubChem) and consulted in the following process. Individual peaks were assigned one chemical formula suitable for their mass and charge. Peaks with an identical formula were bunched into one composition. All compositions were classified into categories that had been pre-defined with key words to be matched, where each composition was allowed to belong to more than one category. Then the distribution of compositions over the categories were visualized as heat maps.

2.5 Statistical Procedure. When assembling peaks into one composition, the intensities of the peaks were totalized and regarded as the intensity of the composition. For N samples and *M* compositions, the intensity (*Vij*) of the *j*-th composition of the *i*-th sample was normalized against the total of intensitives of the sample to the intensity ratio (*Rij*) and further standardized to Z-score (*Zij*) using the mean and standard deviation for the composition as follows:

$$Rij = Vij/\sum_{j=1}^{N} Vij \quad (1)$$

$$Zij = \left(Rij - Ej(Rij)\right)/\sigma j\,(Rij) \quad (2)$$

$$Ej(Rij) = \sum_{i=1}^{M} Rij/M\,,\ \sigma j(Rij) = \sqrt{\frac{\sum_{i=1}^{M}(Rij - Ej(Rij))^2}{M-1}} \quad (3)$$

3. RESULTS AND DISCUSSION

3.1. Identification of compositions

For six saffron samples listed in Table 1, total 10137 peaks of exact mass <1500 were traced through mass spectrometry and deduced to 5310 compositions, of which 3153 compositions were successfully identified.

For the 5310 compositions, the means and standard deviations of their intensity ratios among the samples are plotted in Fig. 1. Led by crocetin with the maximum mean value 0.052770, 6-oxocineole, safranal, dihydrocordoin, dicrocin, rutin, picrocrocin and crocin were in the top group with the mean value > 0.010000 as colorfully marked and listed in the legend. Sucrose showed the maximum standard deviation value 0.009913; its mean value 0.007103 positioned itself not far from the top group. The second largest standard deviation value was 0.006863 of 6-oxocineole. Crocetin, dicrocin, picrocrocin, crocin, safranal and 6-oxocineole are terpenes or their derivatives and well known as saffron-specific components. Dihydrocordoin and rutin are flavonoids. The whole compositions are more systematically examined with their chemical, biological and medical properties in the next section.

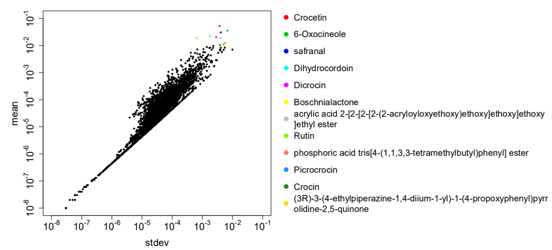

Figure 1. Compositions of six saffron samples. For 5310 compositions, the standard deviation values were plotted to the mean values.

3.2. Classification of compositions

We classified the compositions into 54 categories (C1-54), allowing one composition to appear in more than one category, as shown in Fig. 2. Categories C1-6 were defined for chemical composition, C7-13 and its subcategories C14-29 for nutritional and medicinal aspects, C30-40 for drugs/agents involved in agriculture, environment and medicine, C41-50 for medically affective regions, C51-53 for biological mechanisms, and C54 for unclassified compositions.

For each sample, the intensity ratios of compositions belonging to each category were summed up and heat-mapped in the logarithmic scale in Fig. 2A. For the samples, their category-wise subtotals were standardized to Z-scores and heat-mapped in Fig. 2B.

Obviously in Fig. 2A, almost all are organic compounds and their majority are bunched in C7 (Organic acids, aldehydes, ketones & alcohols) that is a rather widely defined category. In the categories C7-13, C12 (Lipids) is the most populated, followed by C10 (Amino acids & peptides) and C13 (Phytochemicals) and C8 (Carbohydrates). In the subcategories C14-29, C14(Fatty acids & acyls) and C21(Terpenoids) are distinctive. Note that C21 includes terpenes, terpene alcohols and their esters in addition to terpenoids. C30 (Drugs) is also highly populated. Especially, C37 (Anesthetics, Analgesics & Anti-inflammatory drugs) is notable.

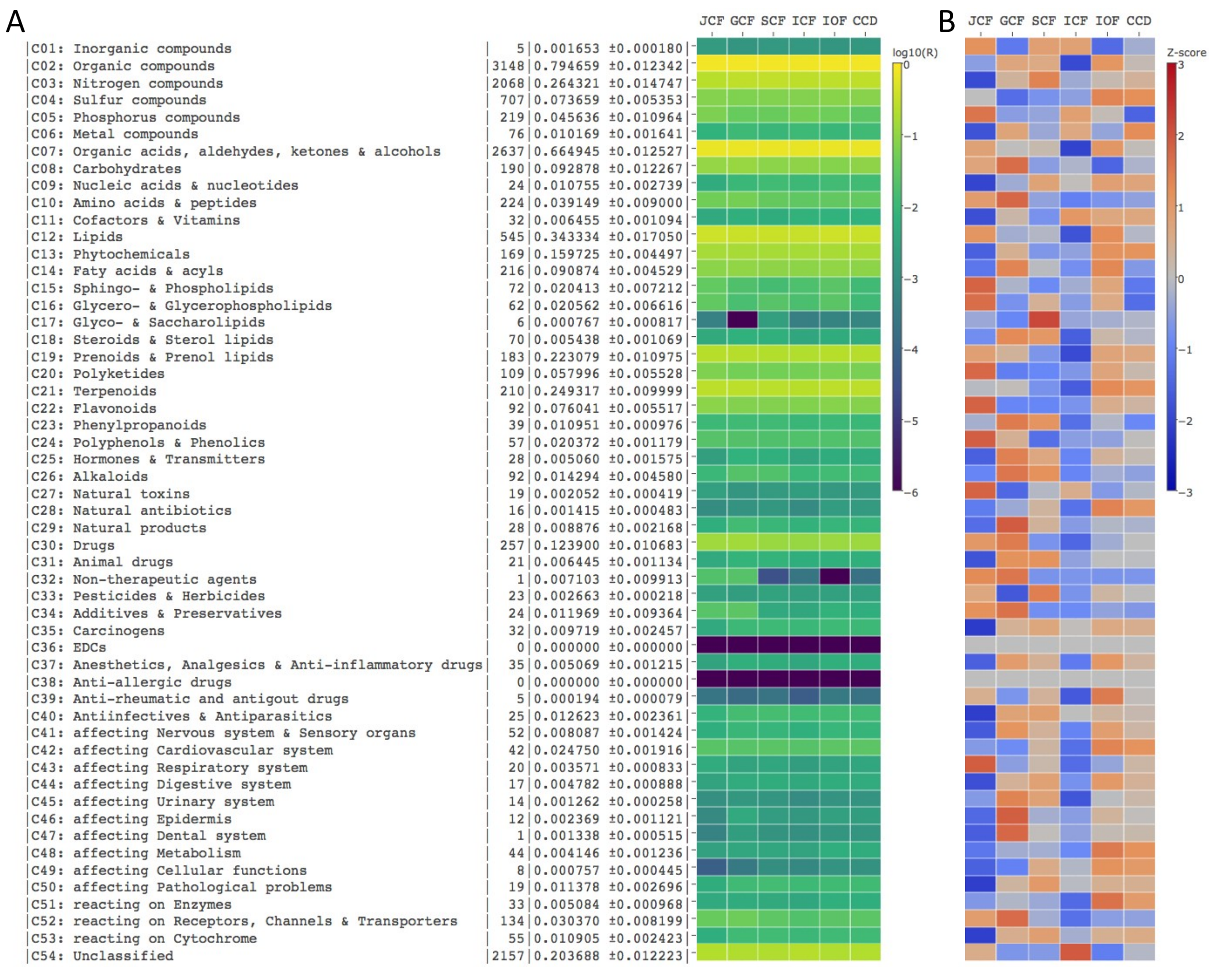

Figure 2. Classification of saffron compositions. (A) For each sample, the subtotals of intensity ratios of compounds belonging to each category are summed up and heat-mapped in the logarithmic scale. Each category name is followed by the number of compositions, the mean and standard deviation of the subtotals. (B) The subtotals are standardized to Z-scores among the samples and heat-mapped.

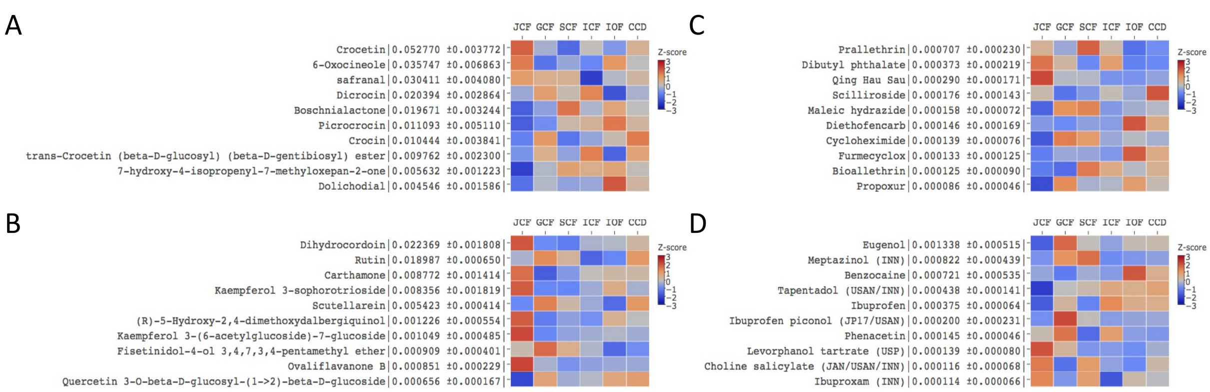

Figure 3. Representative saffron compositions. For four categories (A) C21:Terpenoids, (B) C22:Flavonoids, (D) C33:Pesticides & Herbicides and (D) C37:Anesthetics, Analgesics & Anti-inflammatory drugs, Z-scores of top 10 compositions (in the mean value of intensity ratios) are heat-mapped, where each composition is specified with its name and the mean and standard deviation of intensity ratios of six samples.

For four representative categories, including C21 (Terpenoids), C22 (Flavonoids), C33 (Pesticides & Herbicides) and C37 (Anesthetics, Analgesics & Anti-inflammatory drugs), their top 10 compositions (in the mean value of intensity ratios) are shown in Fig. 3. The former two categories are related with the chemical structure, while the latter two with the agricultural/environmental or medical function. For the latter two categories, their compositions were successfully caught even for such tiny intensity ratios as the order of magnitude -6.

3.3. Relationships of samples

We finally compare the samples. To quantify the compositional similarities between the samples, we conducted Ward cluster analysis, showing the resulting dendrograms in Fig. 4.

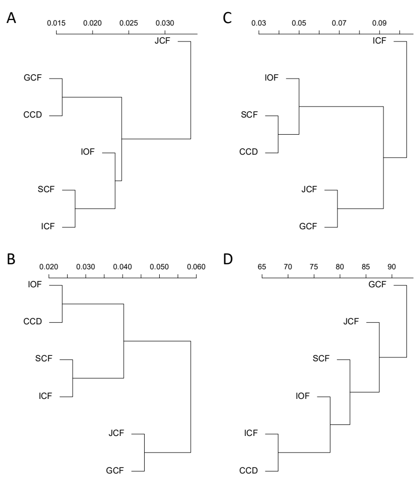

Figure 4. Relationships of six saffron samples. Ward cluster analysis was conducted on (A) the intensity ratios of top 12 compositions, (B) all 5310 compositions, (C) the subtotals of 54 categories, and (D) the Z-scores of all 5310 compositions.

The intensity ratios of the top 12 compositions (Fig. 1) were subjected in Fig. 4A, and the whole 5310 compositions in Fig. 4B. When increasing the number of compositions, top 26 or more compositions gave the same shape of tree as in Fig. 4B. The mean value of the 26th composition was 0.006596, while the maximum was 0.052770. Thus, merely two digits were effective. Fig. 4C is a dendrogram on the subtotals of intensity ratios of compositions of 54 categories. In both Fig. 4B and Fig. 4C, JCF and GCF are separated from the others.

To eliminate the effect of magnitude and reflect the variation of minor compositions, such as pesticides and anesthetics, the Z-scores of the whole compositions were subjected in Fig. 4D. JCF and GCF were placed as the nearest neighbors again, consistently showing their compositional similarity.

In summary, saffron contains a large variety of compositions. From major compositions (e.g., terpenoids and flavonoids) to minor ones (e.g., pesticides and anesthetics), their intensities are variable in a wide range. High precision in mass spectrometry and accuracy in identification and classification of compositions are both essential for assessment of food safety and medical applicability.

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