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# Chromatographic fingerprint analysis of secondary metabolites in citrus fruits peels using gas chromatography–mass spectrometry combined with advanced chemometric methods

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ABSTRACT

Multivariate curve resolution (MCR) and multivariate clustering methods along with other chemometric methods are proposed to improve the analysis of gas chromatography–mass spectrometry (GC–MS) fingerprints of secondary metabolites in citrus fruits peels. In this way, chromatographic problems such as baseline/background contribution, low S/N peaks, asymmetric peaks, retention time shifts, and co-elution (overlapped and embedded peaks) occurred during GC–MS analysis of chromatographic fingerprints are solved using the proposed strategy. In this study, first, informative GC–MS fingerprints of citrus secondary metabolites are generated and then, whole data sets are segmented to some chromatographic regions. Each chromatographic segment for eighteen samples is column-wise augmented with $m/z$ values as common mode to preserve bilinear model assumption needed for MCR analysis. Extended multivariate curve resolution alternating least squares (MCR-ALS) is used to obtain pure elution and mass spectral profiles for the components present in each chromatographic segment as well as their relative concentrations. After finding the best MCR-ALS model, the relative concentrations for resolved components are examined using principal component analysis (PCA) and $k$-nearest neighbor (KNN) clustering methods to explore similarities and dissimilarities among different citrus samples according to their secondary metabolites. In general, four clear-cut clusters are determined and the chemical markers (chemotypes) responsible to this differentiation are characterized by subsequent discriminate analysis using counter-propagation artificial neural network (CPANN) method. It is concluded that the use of proposed strategy is a more reliable and faster way for the analysis of large data sets like chromatographic fingerprints of natural products compared to conventional methods.



## 1. Introduction

The awareness of consumers concerning the relation between food and health is revolutionizing in recent years. This claim is confirmed by ever-increasing uses of natural products in human life [1–4]. Citrus fruits are among the most widely used natural products in the world due to the presence of bioactive compounds, such as phenols, vitamin C and carotenoids [2,5]. However, citrus fruits are also sources of essential oils (EOs) due to their aromatic secondary metabolites which usually obtained from the peels of sweet oranges (*Citrus sinensis* L.), bitter oranges (*Citrus aurantium* L.), lemons (*Citrus limon* L.), bergamots (*Citrus bergamia*), mandarins (*Citrus deliciosa* Ten.) and grapefruits (*Citrus paradise* L.) [1,6,7]. Citrus EOs have been classified as generally recognized as safe (GRAS) due to their antimicrobial, antifungal, antioxidant, anti-inflammatory and anxiolytic activities [1,2,5,7–10]. Due to their great importance, numerous investigations have been performed aimed at identifying the chemical composition of EOs from peels and leaves of different citrus species [6,11–18]. However, the composition and flavor quality of citrus fruits considerably depend on their cultivar, maturity, genotype, origin, climate, season and ripening stage. By considering all the differences in citrus EOs composition, it is clear that only a detailed knowledge of their secondary metabolites will lead to a proper application of their components. However, such a detailed knowledge can be only obtained by means of applying suitable extraction techniques and carefully performed chromatographic analysis [19]. Among different methods, gas chromatography combined to mass spectrometry (GC–MS) is the primary choice for the analysis of citrus EOs [6,19].

On the other side, there has recently been substantial growth of interest in characterization of chemical components of a sample

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using its chromatographic fingerprint which is a chromatogram that represents the chemical characteristics of herbal medicine. In general, samples with similar chromatographic fingerprints have similar properties [20–23]. As a result, chromatographic fingerprint analysis has potential to determine the identity, authenticity, quality, and lot-to-lot consistency of natural products [20–23].

Chromatographic fingerprints of secondary metabolites of citrus EOs usually contain a large number of components and form a very complicated system. In addition, presence of different chromatographic problems, such as baseline drift, spectral background, low signal-to-noise (S/N) ratios, retention time shifts, asymmetric peaks and co-elution (overlapped or embedded peaks), makes isolation of all detectable components much more difficult [24–26]. Multivariate chemometric methods can overcome these problems and extract informative chromatographic and spectral information from second-order data obtained from the GC–MS analysis of citrus EOs [19,24–27].

In the present contribution, a new strategy based on chemometric methods was proposed for the comprehensive chromatographic fingerprint analysis of citrus EOs. First, GC–MS was used to generate the informative chromatographic fingerprints under the optimum analytical conditions. Next, extended multivariate curve resolution-alternating least squares (MCR-ALS) [28–33] along with other chemometric methods were used to solve the common chromatographic problems occurred during the GC–MS analysis of citrus EOs. This would result in obtaining pure elution and mass spectral profiles for the secondary metabolites exist in citrus EOs as well as their relative concentrations. Then, principal component analysis (PCA) [34,35] and *k*-nearest neighbor (KNN) [36] as common unsupervised clustering methods were used to represent changes in chemical compositions of secondary metabolites of citrus EOs among different samples. Finally, the chemical markers (chemotype) which have the most contribution to separated clusters were determined using counter-propagation artificial neural network (CPANN) method [37–39].

## 2. Experimental

### 2.1. Sample collection and chemicals

Citrus fruits were collected randomly from healthy trees of lemon (*C. limon*), orange (*C. Sinensis*), mandarin (*Citrus reticulata*) and grapefruit (*Citrus paradisi*), cultivated under the same pedoclimatic and cultural conditions in an experimental orchard. Random sampling means that among different trees belong to the same species, three samples are selected. For this reason, different fruits were picked up and then a composite sample was chosen for analysis. The orchard was located at the Darab city in Fars province of Iran. Trees were in good vigor, disease free and without visible insect infestation.

Eighteen cultivars (Table 1) were selected and their peels were analyzed. Each sample was analyzed three times.

Normal hexane and anhydrous sodium sulfate (purity > 95%) were purchased from Merck (Darmstadt, Germany). Normal alkanes standards ($C_7$–$C_{25}$) were purchased from Ultra Scientific (North Kingstone, USA).

### 2.2. Extraction of secondary metabolites of citrus fruits

In separate experiments, 50.0 g of air dried peel samples were cut into small pieces and then were completely immersed in water and hydro-distilled in a full glass Clevenger-type apparatus. The extraction was carried out for 3 h. When the condensed material cooled down, the water and essential oils were separated. The oil was decanted to be used as essential oil. To improve the recovery, the essential oil was taken up in n-hexane, dried over anhydrous sodium sulfate until the last traces of water were removed and stored in a dark and air-tight sealed vial at 4 °C and then 0.5 μL of each sample was used for GC–MS analysis.

**Table 1**
Citrus samples included in this study.

| Sample | Name | Abbreviation |
|---|---|---|
| 1 | Lemon 1 | L1 |
| 2 | Lemon 2 | L2 |
| 3 | Lemon 3 | L3 |
| 4 | Lemon 4 | L4 |
| 5 | Lemon 5 | L5 |
| 6 | Lemon 6 | L6 |
| 7 | Lemon 7 | L7 |
| 8 | Lemon 8 | L8 |
| 9 | Orange 1 | O1 |
| 10 | Orange 2 | O2 |
| 11 | Orange 3 | O3 |
| 12 | Orange 4 | O4 |
| 13 | Orange 5 | O5 |
| 14 | Mandarin 1 | M1 |
| 15 | Mandarin 2 | M2 |
| 16 | Mandarin 3 | M3 |
| 17 | Grapefruit 1 | G1 |
| 18 | Grapefruit 2 | G2 |

### 2.3. GC–MS conditions

GC–MS analyses were performed using a HP-6890 GC system coupled with a 5973 network mass selective detector (MSD) and equipped with a HP5-MS capillary fused silica column (60 m, 0.25 mm I.D., 0.25 μm film thickness). The oven temperature program initiated at 40 °C, held for 1 min then rose at 3 °C/min to 250 °C, held for 20 min. Other operating conditions were as follows: carrier gas, He (99.999%), with a flow rate of 1 mL/min; injector temperature, 200 °C; split ratio of 1:20. An oil sample of 0.5 μL was injected in the split mode injection. Mass spectra were taken at 70 eV. The *m*/*z* values were recorded in the range of *m*/*z* 20–350 amu.

### 2.4. Identification of secondary metabolites

The identification of individual components was based on (i) comparison of mass spectral fragmentation patterns with those stored in the NIST Mass Spectral Library built up using pure substances and the mass spectra from the literature and (ii) comparison of the GC retention indices (RIs) on HP-5MS columns, determined relative to the retention time of a series of n-alkanes with linear interpolation, with those of authentic compounds (using a homologous series of n-alkanes ($C_7$–$C_{25}$) in this work) and literature data.

### 2.5. Chemometric analysis

Fig. 1 shows the general framework of the strategy proposed in this work for the comprehensive chromatographic fingerprint analysis of secondary metabolites of citrus fruits. First, the data for each sample was exported into the MATLAB environment as comma separated values (CSV) file. The total ion chromatograms (TICs) of EOs are very complicated due to the large number of constituents of these mixtures. In addition, the efficiency of MCR techniques is increased when the number of chemical components and artifacts like baseline drift and noise remain under certain level in a data matrix. It means when more components included in the data matrix, the probability of occurrence of artifacts in chromatographic and mass spectrometric dimensions increases. Therefore, the overlaid TICs for eighteen samples were segmented to desired number of chromatographic segments using local rank analysis