Authenticity Assessment of Fruit Juices using LC-MS/MS and Metabolomic Data Processing

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Overview

Liquid Chromatography coupled to tandem Mass Spectrometry (LC-MS/MS) with a SCIEX 4000 QTRAP® system was used for comprehensive fingerprinting of several fruit juices. Metabolomic data processing tools were used for authentication, i.e. classification of juices and adulteration detection. The developed statistical model was able to reliably detect 25% of orange juice adulteration with apple or grapefruit juice. In addition, high resolution and accurate mass MS and MS/MS measurements using the SCIEX TripleTOF® 5600 System were performed to identify characteristic markers for fruit juice authenticity.

Introduction

The production of fruit juices represents an important and rapidly growing branch of the beverage industry. Besides orange juice, which is produced and consumed in the largest volume worldwide, other fruit juice types, such as those obtained from pomegranate and various types of berries, have become popular because of high levels of antioxidants resulting in positive health effects. Similarly to other highly prized food commodities, the economic value and large-scale production of juice made them a likely target for adulteration and fraud. The most frequent profit-driven fraudulent procedures applied, either alone or in combination, are dilution with water, addition of sugars or pulp wash, and extension of authentic juice with cheaper alternatives.[1, 2]

As the adulteration of fruit juices represents an ongoing problem, suitable analytical methods are needed to control authenticity parameters dictated by the legislation (Council Directive 2001/112/EC 2001). Until now, a number of methods have been developed to tackle various aspects of fruit juice authenticity. The most established approaches are based on profiling of carbohydrates, phenols, carotenoids, amino acids, or other organic acids using different chromatographic and spectroscopic methods.[3-5]

All of these methods are of targeted nature and can only be used to monitor one or few specific adulteration practices. However, it should be noted that fraud performers are usually one step ahead of the available testing methods, as new and more sophisticated adulteration practices are continuously developed. Therefore, analytical approaches for more comprehensive insight into chemical composition of fruit juices and its changes associated with adulteration are needed.

The field of metabolomics, a systematic study of the unique chemical fingerprints of samples, has recently found its application in many research areas including food quality and authenticity assessment. Advanced data mining tools are required to process and interpret complex data obtained within metabolomic-based studies.[6]

In this study, the feasibility of LC-MS/MS techniques employing QTRAP and TripleTOF systems for metabolomic-based authentication of fruit juices (including apple, blueberry, cranberry, grapefruit, orange, pomegranate, and their mixtures) was explored. Complex LC-MS/MS data were processed using Principal Components Analysis (PCA), Principle Components Variable Grouping (PCVG), and Linear Discriminant Analysis (LDA) to assess the suitability of the data to differentiate juice types and to detect their adulteration. In addition, high resolution and accurate mass MS and MS/MS data were acquired for characteristic marker compounds to empirically calculate their elemental formulas and for tentative identification.[7]

Experimental

Sample Preparation

Different fruit juices made of apple (n = 16), blueberry (n = 1), cranberry (n = 1), grapefruit (n = 16), orange (n = 19), and pomegranate (n = 1) were purchased from Czech and Canadian supermarkets. Collected samples represented both freshly squeezed juices and juices prepared from concentrate, and were produced in various countries. Mixtures of different juices were prepared in various ratios to simulate adulteration.

Fruit juice samples were centrifuged to remove solid particles, 100x diluted, and transferred into autosampler vials for analysis.

LC

LC separation was achieved using an Agilent 1200 LC system with a Restek Ultra Aqueous C18 column (50 x 2.1 mm, 3 µm) and a gradient of water with 5 mM ammonium acetate and methanol with a total run time of 10 min. The injection volume was set to 10 µL.

MS/MS

The SCIEX 4000 QTRAP® System equipped with Turbo V™ source and electrospray ionization (ESI) probe was used for metabolomic fingerprinting of juice samples. Full scan MS was acquired in EMS mode over a mass range of 100 to 1000 amu using dynamic fill time to avoid possible ion trap saturation for highly abundant compounds while enhancing sensitivity for compounds present at low concentrations. Information dependent acquisition (IDA) was used to automatically acquire MS/MS data when an MS signal exceeded a threshold of 3000 cps. The collision energy was set to 35 V with a spread of ±15 V.

Marker compounds were tentatively identified by processing MS and MS/MS data acquired using the SCIEX TripleTOF® 5600 System. The system was operated with the DuoSpray™ source. The ESI probe was used for sample analysis and the APCI probe was used to perform automatic mass calibration through the calibrant delivery system (CDS). TOF-MS (100 ms) and TOF-MS/MS (50 ms) acquisition were combined in an IDA method.

Results and Discussion

Chemometric Analysis

Juice samples were analyzed by LC-MS/MS in randomized order to avoid any possible effect of time-dependent changes in chemical fingerprints. Full scan MS chromatograms were processed using PCA and PCVG in MarkerView™ software.

PCA finds combinations of variables that explain the variance present in the data set. For each principal component (PC), every sample has a score, and every variable has a loading that represents its contribution to the combination. It is common practice to plot the scores and loadings for two PCs to visualize results and to identify characteristic marker compounds.

The scores plots for different juice samples analyzed in negative and positive polarity are displayed in Figures 1a and 1b. PCA of the data set revealed three separate clusters of apple, orange, and grapefruit juices samples, showing differences in LC-MS profiles associated with the fruit type. However, it is apparent that more pronounced clustering and significantly better resolution among sample clusters were obtained for positive ionization data. Therefore, only data recorded in positive ionization mode were further used in this study.

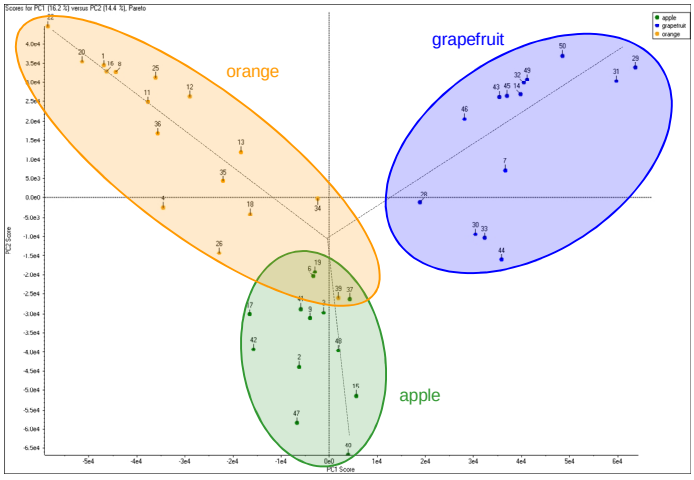

Figure 1a. Scores plot of PCA of apple, orange, and grapefruit juice samples analyzed using negative polarity LC-MS/MS

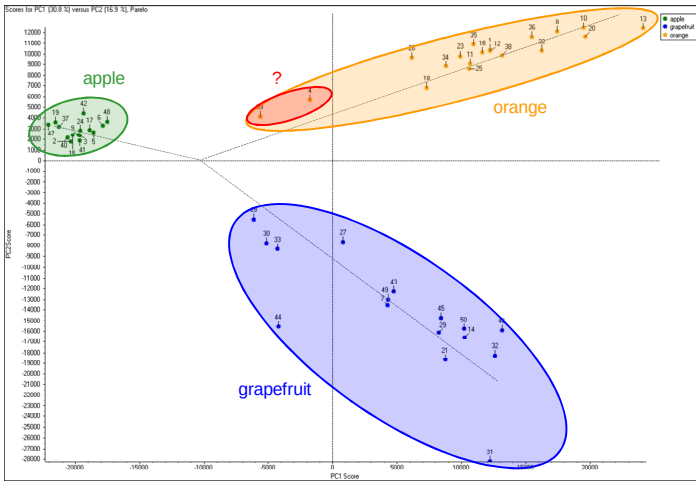

Figure 1b. Scores plot of PCA of apple, orange, and grapefruit juice samples analyzed using positive polarity LC-MS/MS

Note that two orange juice samples (# 4 and # 39) are located slightly separated from the main cluster of all other orange juice samples. This indicates a potential adulteration of these two juice products.

The corresponding loading plot in Figure 2 shows the variables that make the most difference in separating juice samples. It can be used to identify the molecular ion and retention time of characteristic marker compounds.

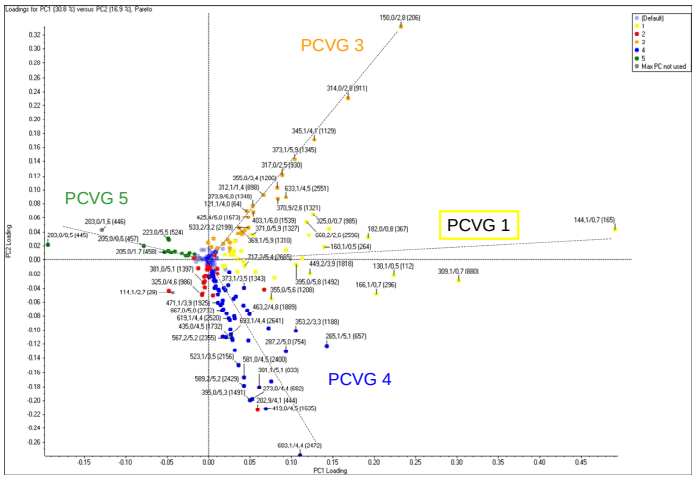

Figure 2. Loadings plot of PCA after PCVG of apple, orange, and grapefruit juice samples analyzed using positive polarity LC-MS/MS showing identified marker ions (m/z, retention time pairs)

Characteristic marker compounds of a group of samples are located in the same area of the loadings plot as the group is located in the scores plot. PCVG was utilized to automatically group variables to facilitate data interpretation. Four characteristic groups of variables were identified to be responsible for clustering of samples representing respective fruit juice types (apple group 5, grapefruit group 4, orange group 3, and all citrus fruits group 1).

Characteristic marker ions (m/z, retention time pairs) can be displayed in profile plots to verify the unique occurrence of marker ions in tested juice samples. Selected marker ions, i.e. 203 at 0.5 min for apple, 603 at 4.4 min for grapefruit, 633 at 4.5 min for orange, and 130 at 0.5 min, 144 at 0.7 min, and 160 at 0.5 min for citrus fruits are shown (Figure 3).

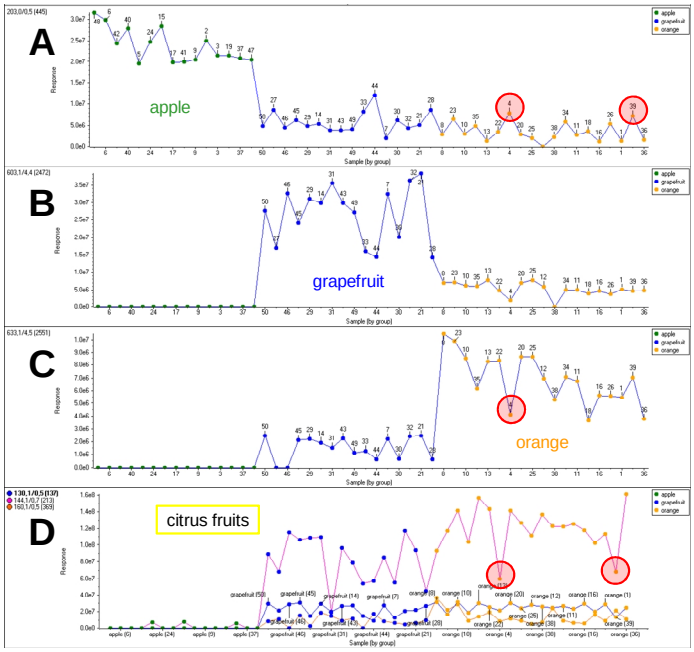

Figure 3. Profile plots of six selected marker ions for A) 203 at 0.5 min for apple, B) 603 at 4.4 min for grapefruit, C) 633 at 4.5 min for orange, and D) 130 at 0.5 min, 144 at 0.7 min, and 160 at 0.5 min for citrus fruits

The suspicious and potentially adulterated orange juice sample show slightly higher levels of the apple juice marker and lower level of characteristic markers for orange and citrus.

In a next step, the detection of adulterated orange juice was quantified by comparing laboratory prepared mixtures of orange juice with apple juice and grapefruit juice at different adulteration levels (Figures 4a and 4b). The LDA statistical model constructed with the use of statistiXL software (Nedlends, WA, Australia) was able to reliably detect 25% of orange juice adulteration with apple or grapefruit juice. Both recognition and prediction abilities of the model were 100%.[7]

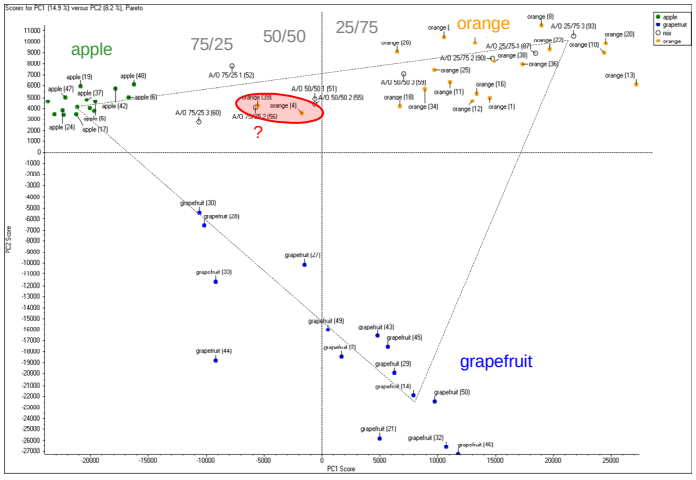

Figure 4a. Scores plot of PCA of apple, orange, and grapefruit juice samples and mixtures of apple and orange juice

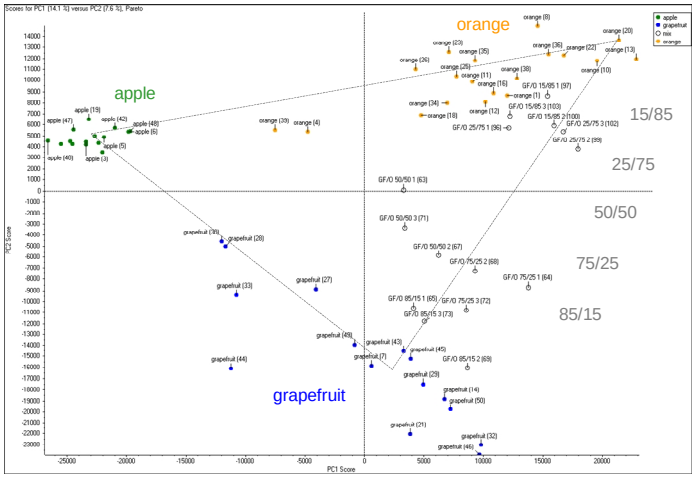

Figure 4b. Scores plot of PCA of apple, orange, and grapefruit juice samples and mixtures of grapefruit and orange juice

The model suggests an adulteration level of the suspicious orange juice samples (# 4 and # 39) of approximately 50% with apple juice (Figure 4a).

A similar experiment was carried out for other types of fruit juices, including apple, blueberry, cranberry, pomegranate, and their mixtures to simulate adulteration.

The loadings plot presented in Figure 5 shows that PCA can separate between these types of juices. However, only one juice sample was available per fruit type. Thus, the data set does not reflect the natural variability of the investigated fruits, but proves that LC-MS/MS with metabolomic processing seems to be applicable to these fruit juice types.

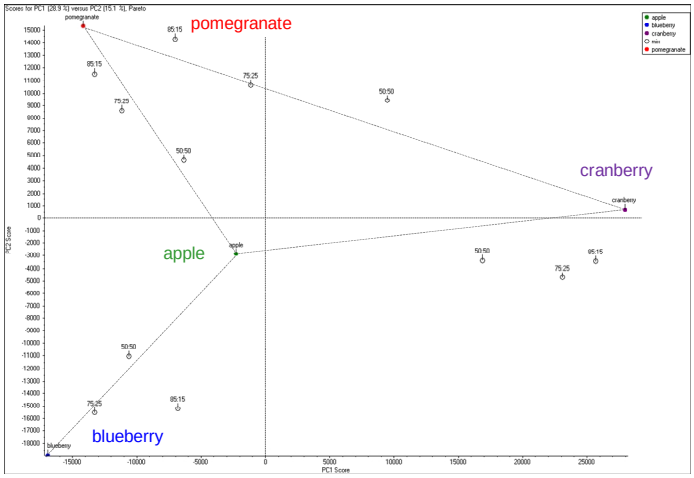

Figure 5. Scores plot of PCA of different juice samples and their mixtures

Tentative Identification of Marker Compounds

The identification of characteristic Marker compounds represents the most laborious and time-consuming step of the metabolomic workflow. Accurate mass MS and MS/MS measurements using the SCIEX TripleTOF® 5600 system were performed and data were processed using PeakView® Software (version 1.2) to empirically calculate molecular formulas and to automatically perform online database searching for potential structures.

The formula finder uses high resolution accurate mass information of the molecular ion, adducts, isotopic pattern, and fragment ion information to empirically calculate potential molecular formulas for the detected compound. Furthermore, the calculated formulas are then automatically searched against online databases, like PubChem, Nist, and ChemSpider, to find possible matching structures.

The examples presented in Figures 6a and 6b show the tentative identification of the characteristic marker ions for orange (633 at 4.5 min) and grapefruit (603 at 4.4 min) as the flavones glycosides hesperidin and naringin. In both cases the molecular ion was automatically identified as Na-adduct.

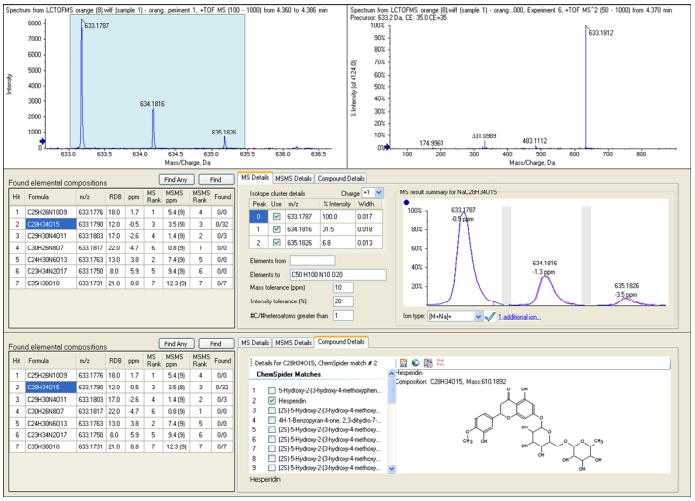

Figure 6a. Tentative identification of a marker ion characteristic for orange (633 at 4.5 min) as hesperidin based on empirical formula finding and automatic online database searching

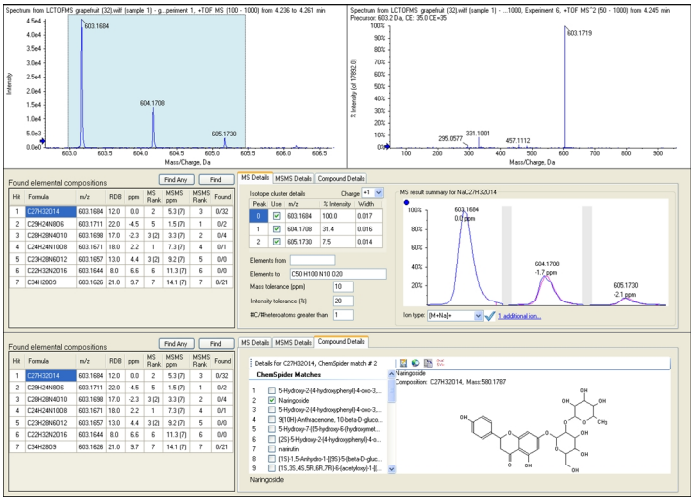

Figure 6b. Tentative identification of a marker ion characteristic for grapefruit (603 at 4.4 min) as naringin (naringoside) based on empirical formula finding and automatic online database searching

PeakView® Software also allows comparing structures (imported mol-file obtained from online database search) with accurate mass MS/MS information to further increase confidence in identification.

The characteristic marker ions for citrus fruits (130 at 0.5 min, 144 at 0.7 min, and 160 at 0.5 min) were tentatively identified as N-methylproline, N,N-dimethylproline, and hydroxyl-N,N-dimethylproline. Figure 7 shows screenshots of the fragment prediction tool of PeakView® Software. In all cases, 100% of the fragment ion intensity of the MS/MS of the precursor ion 130, 144, and 160 were explained by the structures of N-methylproline, N,N-dimethylproline (proleine betaine), and hydroxyl-N,N-dimethylproline (betonicine), respectively. The presence of betaines in citrus fruits was previously reported by Servillo et al.[8]

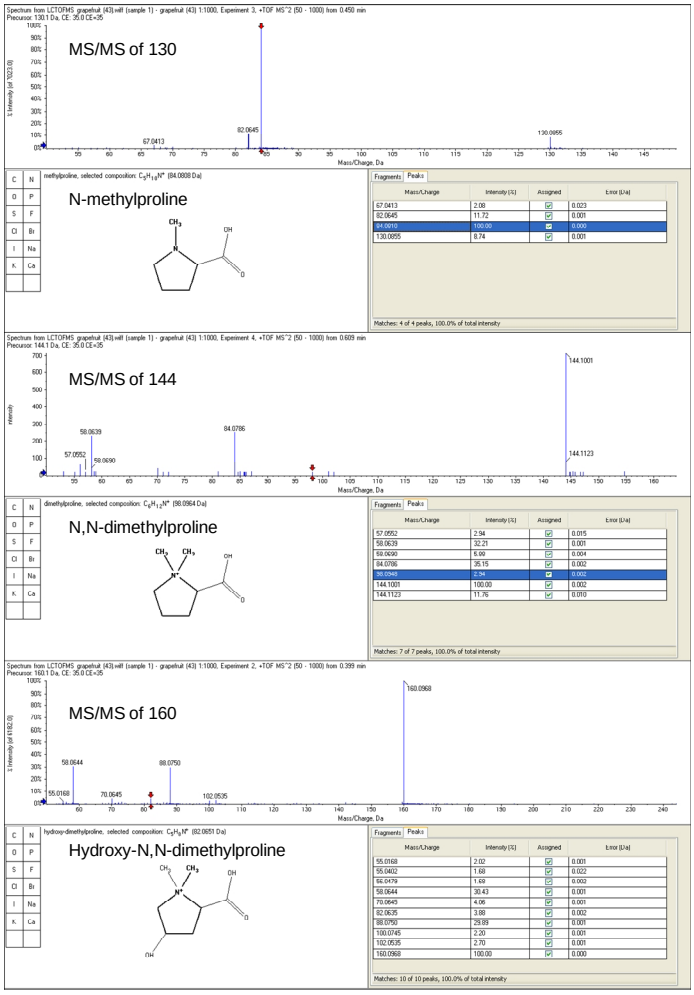

Figure 7. MS/MS fragment ion prediction for N-methylproline, N,N-dimethylproline, hydroxy-N,N-dimethylproline, 100% of MS/MS ions are explainable supporting the tentative identification in citrus fruits

The most characteristic marker compounds for apple juice were tentatively identified as hexose ($C_6H_{12}O_6$) and sugar alcohol ($C_6H_{14}O_6$). However, the existing LC-MS/MS data do not allow discrimination between the isomeric species of sugars and sugar alcohols.

Summary

Comprehensive, non-target LC-MS/MS with metabolomic data processing was demonstrated to be a powerful tool for fruit authenticity assessment. The SCIEX 4000 QTRAP® System was used to collect information-rich full scan data to discriminate different juices using statistical processing with PCA and PCVG. It was possible to reliably detect orange juice adulteration of 25% with apple or grapefruit juice. The feasibility of this approach for authentication of other highly prized fruit juices, such as pomegranate, blueberry or cranberry, was also shown.

Finally, characteristic marker compounds for each juice, pre-selected during PCA, were tentatively identified by processing of accurate mass MS and MS/MS data generated with a SCIEX TripleTOF® 5600 System. The formula finder integrated into PeakView® Software automatically evaluates accurate mass information of the molecular ions, the isotopic pattern, adducts, and MS/MS fragment ions. Resulting molecular formulas are automatically searched against online databases to find matching chemical structures.

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