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ORIGINAL PAPER

HPLC–PDA/ESI–MS/MS detection of polymethoxylated flavones in highly degraded citrus juice: a quality control case study

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Abstract Highly degraded citrus fruit juices collected during a quality control investigation of the Italian Ministry of Agricultural Alimentary and Forestry Policies (MIPAAF) were analyzed for verifying their technological history. The analyzed samples were fermented black fluids stated as degraded first strength juices, in order to justify communitarian aids. Polymethoxylated flavones (PMFs) detection of solid phase extracted (SPE) purified samples provided clean chromatograms and good separation of compounds of interest using high performance liquid chromatography coupled with diode array and mass spectrometry HPLC–PDA/ESI–MS/MS. Since the PMFs concentration is elevate in peel flavedo, it is directly correlated with juice extraction strength. Results showed that all analyzed samples presented a PMFs concentration ranging from two to eight times first strength juices, not respecting the minimum quality requirements of the European regulatory framework.

Keywords Citrus juices · Genuine juices · PMF · Quality control · Mass spectrometry

Introduction

The European citrus fruit sector is strongly orientated toward growing for the fresh produce market. Fruit for processing into juice is mainly fruit rejected during grading, fruit of low quality and sometimes fruit stemming from overproduction. Fruit for processing is usually only an additional source of income for producers. Council Regulation (European Economic Community) No 2601/69 introduced aid for the processing of oranges into juice. Mandarins, satsumas and clementines were incorporated into this scheme in 1989. In 1996, Council Regulation (European Community) No 2200/96 introduced a major reform of the Common Market Organization. Among other things, it gave a central role to the Producer Organisations. The reform also included two other Regulations: Regulation (European Community) No 2202/96, which restructures the aid scheme for processed citrus fruit, and Regulation (European Community) No 2201/96, which revises the aid scheme for processing other products and the trade regime with third countries. For citrus fruit intended for processing, the aid still accounts for the major part of producers' incomes and thus undeniably helps to improve these producers' incomes. However, there are minimum quality requirements apply to fruit delivered for processing that must be respected and assessed. A common fraud consists to hide under simulated accidents (i.e., intentional interruptions of refrigeration chain) the production of second strength juices that, once decomposed, were stated as first strength juices.

Fresh fruits and their hand-squeezed or industrially processed juices contain mostly flavonoids such as flavanone-*O*-glycosides (FGs) and flavones [1]. A flavonoid skeleton is composed of two aromatic rings, which are connected through a pyrone ring in the case of flavones, or a dihydropyrone ring in the case of flavanones. The main citrus FGs are narirutin and hesperidin and are mainly contained in the albedo. The polymethoxylated flavones (PMFs) are a class of minor components usually found in the essential oils fraction of citrus peels [2] (Fig. 1). Hand-

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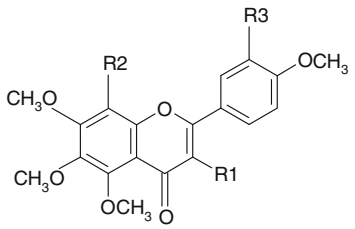

PMF	R_1	R_2	R_3
Sinensetin	H	H	OCH_3
3,5,6,7,3',4'-hexamethoxyflavone	OCH_3	H	OCH_3
Nobiletin	H	OCH_3	OCH_3
5,6,7,4'-tetramethoxyflavone	H	H	H
3,5,6,7,8,3',4'-heptamethoxylflavone	OCH_3	OCH_3	OCH_3
Tangeretin	H	OCH_3	H

Fig. 1 Chemical structure of investigated PMFs

squeezed juices contain a relative low amount of FGs and no detectable traces of PMFs [3]. Commercial juices could be rich in FGs and PMFs because the industrial processing of fruits leads to juices strongly processed and/or contaminated with the peel constituents. Literature reported that the relative distribution of FGs and PMFs could be used to discriminate second extracted juices from first strength ones, because FGs and PMFs concentrations were 4–5 and 20 times higher than in first strength juices, respectively [3]. Although PMFs represent minor constituents in the whole fruit, they occur at high concentrations in peels [1]. Therefore, adulteration of orange juice with peel or pulp wash could be detected from the amount and distribution of PMFs.

This paper reported the results of a quality control investigation performed by the Italian Ministry of Agricultural Alimentary and Forestry Policies (MIPAAF) on highly degraded citrus fruit juices collected from the producers and declared as corrupted first strength juices. The PMFs were isolated, characterized and quantified in 15 samples. Six main compounds (sinensetin, 3,5,6,7,3′,4′-hexamethoxyflavone, nobiletin, 5,6,7,4′-tetramethoxyflavone, 3,5,6,7,8,3′,4′-heptamethoxylflavone, tangeretin) were identified based on their UV spectra, MS data and elution order described in the literature.

Materials and methods

Chemicals A reference solution of sinensetin was purchased from Extrasynthese (Milan, Italy). HPLC-grade acetonitrile, methanol and formic acid were supplied by Romil (Milan, Italy). Distilled water was purified at 18.2 MΩ cm with a MilliQ ULTRA (Millipore, Vimodrone (MI), Italy) purification system.

Standard solutions preparation and storage The working solutions of standard sinensetin were prepared each time by diluting a stock solution in methanol (1,000 mg/L). The stock solution was stored at 4 °C, and in that condition, it was stable for 30 days.

Juices pickup The juices pickup was made by MIPAAF officers from Italian producers. Fifteen concentrated samples (about 60°Brix) of highly degraded citrus juices were collected in Italy in the frame of quality control investigation performed by MIPAAF. For every sample, an official report was written. The samples were labeled with a description of pickup location, pickup date, sample typology and origin and sent to Catania Laboratory of Central Inspectorate for Quality Control of Agricultural and Food Productions (ICQRF) for the analyses. Once analyzed, our laboratory emitted certificate of analysis.

PMFs determination in juice samples PMFs extraction was performed using C_{18} extraction cartridges. The concentrate juices (about 60°Brix) were opportunely diluted with distilled water to 11°Brix, the typical concentration of a single strength orange *juice* when extracted from oranges, and filtered through paper filter (Whatman, VWR International, Milan, Italy). Aliquots of 5 mL were loaded onto the Supelco C_{18} SPE cartridges (Milan, Italy) which were preconditioned successively with 3 mL of methanol and 10 mL of distilled water. The cartridges were washed with 10 mL of distilled water to discharge water soluble interferences and PMFs fraction eluted with 2 mL of methanol. The collected fraction was evaporated to dryness under nitrogen flow, the residue dissolved in 0.5 mL acetonitrile and filtered by 0.45 μm PTFE filters (LabService Analytica, Bologna, Italy). The filtrate was kept at 4 °C until duplicate analyses; three replicate extractions were performed for each sample. The PMFs (sinensetin, 3,5,6,7,3′,4′-hexamethoxyflavone, nobiletin, 5,6,7,4′-tetramethoxyflavone, 3,5,6,7,8,3′,4′-heptamethoxylflavone, tangeretin) were identified on the basis on their UV spectra, MS data and elution order described in the literature. Sinensetin has been quantified using external calibration ($y = 380876x - 5{,}301$; $r^2 = 0.9999$; $n = 5$; $\lambda = 330$ nm), while 3,5,6,7,3′,4′-hexamethoxyflavone, nobiletin, 5,6,7,4′-tetramethoxyflavone, 3,5,6,7,8,3′,4′-heptamethoxylflavone and tangeretin have been determined in terms of their relative amounts.

Chromatographic conditions The analysis was performed with a liquid chromatograph consisting of a Finnigan Surveyor MS-pump, Finnigan Surveyor autosampler and Finnigan Surveyor photodiode-array detector (PDA), coupled with a Finnigan LCQ DECA XP MAX detector

(Thermo Fisher Scientific, Rodano (MI), Italy). The analytical column was a Luna C_{18} 250 × 4.6 mm, 5 μm i.d. (Phenomenex, Anzola Emilia (BO), Italy), the flow rate was 1 mL/min, the column temperature 30 °C and the injection volume 20 μL. Flow rate was split 1/10 before MS interface. A binary gradient of 1% formic acid in water (A) and acetonitrile (B) was employed. The mobile phase gradient was programmed as follows: 0 min, 5% B; 50 min, 28% B; 60 min, 43% B; 60–65 min, 43% B; 70–80 min, 5% B. The range of wavelengths examined by the photodiode-array detector was 190–700 nm, and for quantitative determinations, the chromatograms were recorded at 330 nm. Mass spectral analyses were performed using a LCQ ion-trap mass operating in the positive ion mode using an ion spray LC/MS interface. The electrospray ionization (ESI) needle voltage was 4.0 kV. The capillary voltage was 18 V, and the heated capillary was 250 °C. A sheath gas flow rate of 36 (arbitrary units) was used, and the auxiliary gas was set to 12 (arbitrary units). The PMFs were detected in MS/MS conditions under SRM mode (selected reaction monitoring). Preliminary tuning was carried out with continuous introduction of a dilute solution of sinensetin at the flow rate of 5 μL/min, and the voltages on the lenses were optimized in TunePlus (Excalibur software).

Method validation for sinensetin determination

The method was validated for UV–Vis sinensetin determination and applied to the other PMFs object of study.

Linearity The HPLC/PDA calibration curves were obtained diluting 6 independent stock solutions of sinensetin over the range of about 2.5–130.0 mg/L, making 4 replicates for each concentration. The peak area integration was performed at λ 330 nm, and the relationship with the concentration was determined using linear regression. The regression equation with slope, intercept and coefficient of correlation (r^2) was calculated.

Matrix effect evaluation by standard addition method Six standard solutions of sinensetin in the range 2.5–130.0 mg/L are added to a highly degraded sample juice (unknown) to account for in the calibration any impurities in the unknown, making 4 replicates for each concentration. The point at zero concentration added is the reading of the unknown, and the other points are the readings after adding increasing amounts of standard solution. The absolute value of the x-intercept is the concentration of sinensetin in the unknown. The regression equation with slope, intercept and coefficient of correlation (r^2) was calculated.

Limit of detection (LOD) and limit of quantification (LOQ) At least 7 replicates of aqueous solutions (negative controls) spiked with sinensetin at level closed to the detection limit were prepared, and the standard deviations (σ) were determined. The LOD and the LOQ were obtained as follows:

$$\mathrm{LOD_{INSTR}},\ \mathrm{mg/L} = 3 \times \sigma$$

$$\mathrm{LOD_{INSTR}},\ \mathrm{mg/L} = 10 \times \sigma$$

Method precision (repeatability) Method precision was evaluated by analyzing 10 independent solutions of spiked highly degraded sample juice at 2 different concentration levels (10.0 and 50.0 mg/L). The intraday precision was calculated for each concentration level as RSD (%).

Accuracy (recovery) Accuracy of the method was ascertained by spiking of spiked highly degraded sample juice with a known amount of sinensetin. The spikes were done at 2 different concentration levels (10.0 and 50.0 mg/L) of the estimated sinensetin concentration level of the samples, ten times. The average percentage of recovery (E) at each concentration level was evaluated as follows:

$$E,\ \% = (C \times 100)/\mathrm{TC}$$

where, E = accuracy, C = mean of calculated concentrations and TC = theoretical concentration.

Results and discussion

The FGs and PMFs investigation was made on highly degraded, fermented and black colored fluids with the aim of investigating on their original composition. Many analytical procedures require no preliminary separation of the flavonoid fraction, and analyses are performed directly on the crude juices [4, 5]. Due to the complexity of investigated matrixes, a purification step using SPE cartridge was necessary. The extracts were analyzed using HPLC/PDA/MS detection. A mobile phase gradient with a gradual increase in acetonitrile was chosen to allow a simultaneous and great separation of FGs and PMFs. Nevertheless, the obtained chromatograms did not allow a right quantification of FGs (particularly narirutin and hesperidin), because of the strong matrix effect. On the contrary, the PMFs were resolved in a clean chromatographic region and could be correctly identified and quantified (Fig. 2). The diode array detection and the electrospray mass spectrometry (ESI/MS) were used for a quantitative and qualitative determination of the PMFs content in the samples. HPLC coupled with mass spectrometry in MS/MS was used for the PMFs structural characterization, while their quantification was performed by UV–Vis as sinensetin relative amount. The MS–ESI source is very soft in the ionization; preliminary mass

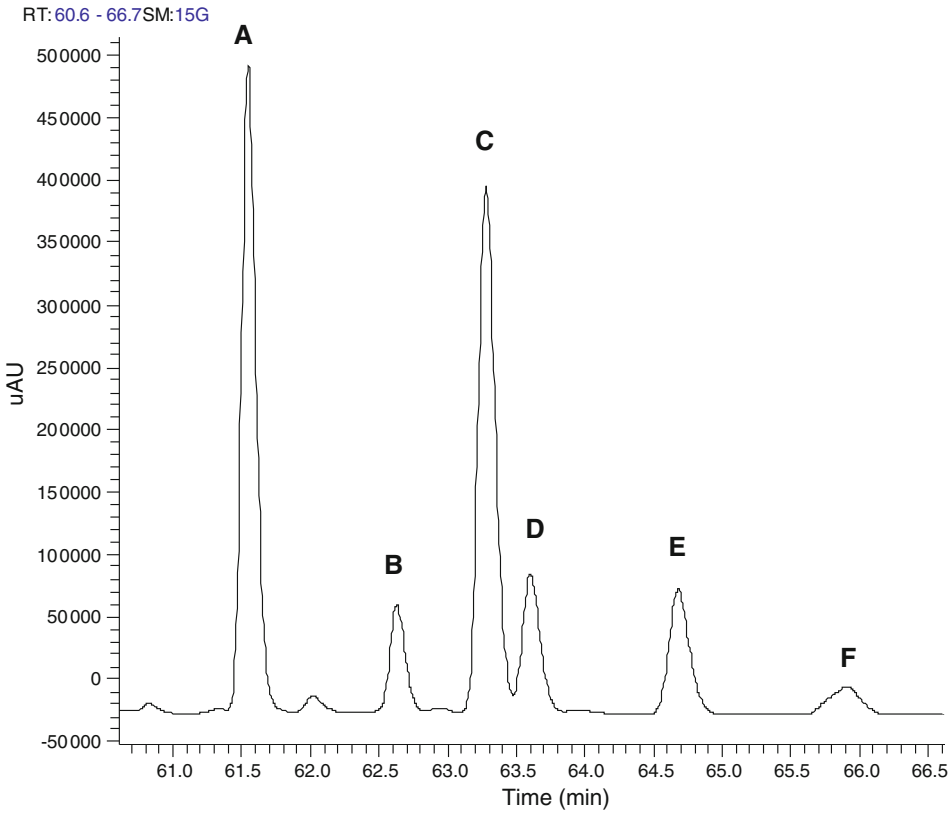

Fig. 2 LC/PDA chromatogram of PMFs in a sample of analyzed orange juice. **a** sinensetin; **b** 3,5,6,7,3′,4′-hexamethoxyflavone; **c** nobiletin; **d** 5,6,7,4′-tetramethoxyflavone; **e** 3,5,6,7,8,3′,4′-heptamethoxylflavone; **f** tangeretin

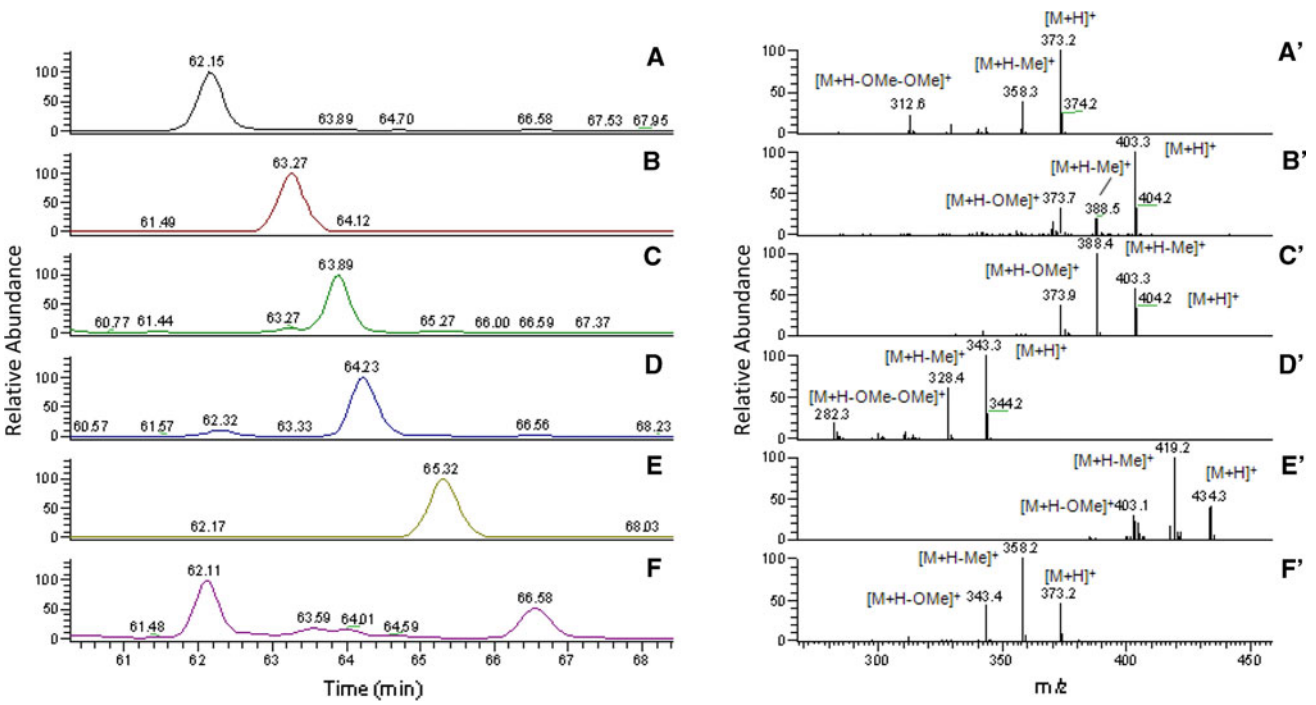

Fig. 3 LC/ESI/MS/MS chromatogram of PMFs in a sample of analyzed orange juice (X) and relative mass spectrum (X′). **a** sinensetin (m/z 312, $ms^2$373@27.00); **b** 3,5,6,7,3′,4′-hexamethoxyflavone (m/z 369, $ms^2$403@27.00); **c** nobiletin (m/z 390, $ms^2$403@27.00); **d** 5,6,7,4′-tetramethoxyflavone (m/z 343, $ms^2$343@27.00); **e** 3,5,6,7,8,3′,4′-heptamethoxylflavone (m/z 419, $ms^2$433@27.00); **f** tangeretin (m/z 358, $ms^2$373@27.00)

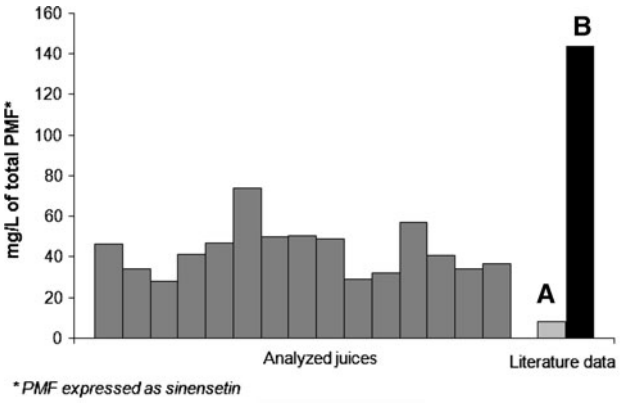

Fig. 4 PMFs content (mg/L) in analyzed juices and mean literature value [3, 8, 9] of PMFs for first (**a**) and second (**b**) strength juices

spectra performed in total ion current (TIC) generated in positive mode showed $[M+H]^+$ ions at m/z 343, 403, 373 and 433 together with other fragments depending on the voltage applied to the source. The parent ions $[M+H]^+$ were further fragmented for confirmatory purposes, and the obtained spectra were reported in Fig. 3. The daughter ions resulted from the loss of CH_3 and CH_3OH. The obtained fragmentation pattern and elution order were in agreement with literature data [6, 7], confirming the PMFs identity.

For what concerning the PMFs amounts in the analyzed samples, the quantitative data were expressed in terms of sinensetin content at λ 330 nm. The regression line performed in presence of matrix with the standard addition methods showed a slope (slope: 381,325; $r^2 = 0.999$) comparable to the corresponding one obtained in solvent ($y = 380876x - 5{,}301$; $r^2 = 0.999$). Value of 0.999 for a six points calibration curves (4 replicates for each point) must be considered proof of the linear relationship. Therefore, we conclude that no matrix effect occurred for sinensetin determination. For what concerning instrument precision evaluation 0.3% could be considered the limit set for the precision of the instrumental system, showing that the equipment used for the study operated correctly for the developed method and produced highly repetitive results. The method showed a mean recovery ranging from 94 to 96% (precision of 4%) for 10 and 50 mg/L spiked concentration level, respectively. The obtained limit of detection (LOD) and the limit of quantification (LOQ) were 0.1 and 0.3 mg/L, respectively.

The PMFs quantitative data showed overall amounts ranging from 27 to 74 mg/L. These data were compared with the contents reported in literature for first (4–12 mg/L) and second strength juices (100–180 mg/L) [3, 8] (Fig. 4): these data demonstrated that the analyzed samples could not be considered first strength orange juices and probably contained peel extracts. Besides, a partial degradation of the original content of PMFs must be considered because of the high degree of degradation of the samples. For what concerning PMFs relative distribution, data showed a dominance of sinensetin and nobiletin with traces of tangeretin, confirming the typical orange juice PMFs pattern (Fig. 5).

Despite their scientifically accepted importance as a marker of citrus technological history [3, 4, 9], PMFs are not usually considered as a quality parameter to investigate in routinely official analysis. The results of this case study demonstrated that PMFs determination could be employed to investigate the industrial practices, even in highly degraded citrus juices. Therefore, it should be considered as an additional parameter to investigate in juice control, in

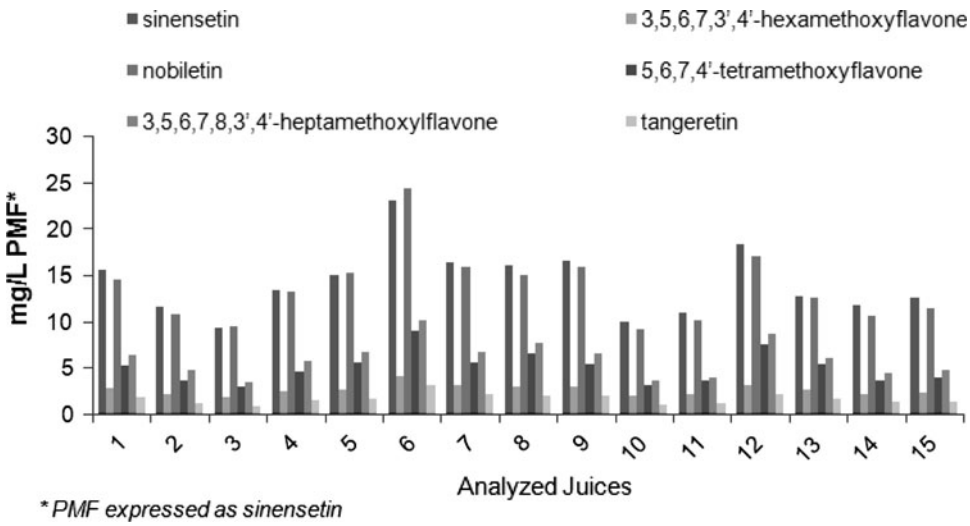

Fig. 5 Relative distribution of PMFs (mg/L) in analyzed orange juices

order to preserve consumers from commercial illicit and safeguard honest manufacturers.

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