The influence of rootstock and extraction setting on the Limonin and Flavonoids levels in orange juice during ripeness

A influência do porta-enxerto e do ajuste de extração nos níveis de Limonina e Flavonóides no suco de laranja durante a maturação

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ABSTRACT
The influence of rootstock, maturity and extraction settings on limonin, hesperidin and narirutin levels of orange juice was evaluated. A liquid chromatographic method to determine limonin was developed and validated. The linear range was 0.410 to 61.5 µg.mL-1, with a linear correlation coefficient higher than 0.999. The limit of detection was 0.144 µg.mL-1and limit of quantification 0.363 µg; precision showed RSD≤5.0% and accuracy was from 92.6 to 100.4%. Limonin was identified in Pêra-Rio orange juices from Cleopatra mandarin and Rangpur lime rootstocks extracted in the NFC and FCOJ settings during the 2013 harvest. Limonin levels in Pêra-Rio orange juices ranged from 0.86 to 3.94 µg.mL-1 and flavonoids hesperidin and narirutin levels ranged from 12.00 to 26.02 µg.mL-1 for narirutin and from 122.12 to 175.01 µg.mL-1 for hesperidin. Principal component analysis was able to differentiate the juices from Cleopatra mandarin and Rangpur lime rootstocks according to ripeness, as well as extraction settings. Limonin and flavonoid levels reduced during maturation. Limonin levels were more expressive at the beginning of the harvest, especially in juice obtained with the FCOJ extraction setting. Hesperidin levels were about ten times higher than narirutin levels in all juices.

Keywords: Limonin, pêra-rio orange juice, HPLC, narirutin, Hesperidin.

RESUMO
Foi avaliada a influência do porta-enxerto, da maturidade e da extração nos níveis de limonina, hesperidina e narirutina do suco de laranja. Foi desenvolvido e validado um método cromatográfico líquido para determinar a limonina. A faixa linear foi de 0,410 a 61,5 µg.mL-1, com um coeficiente de correlação linear maior que 0,999. O limite de detecção foi de 0,144 µg.mL-1 e o limite de quantificação de 0,363 µg; a precisão mostrou RSD≤5,0% e a precisão foi de 92,6 a 100,4%. A limonina foi identificada nos sucos de laranja Pêra-Rio de tangerina Cleopatra e Rangpur, raízes de limão extraídas nos ambientes NFC e FCOJ durante a colheita de 2013. Os níveis de limonina no suco de laranja Pêra-Rio variaram de 0,86 a 3,94 µg.mL-1 e os níveis de flavonóides hesperidina e narirutina variaram de 12,00 a 26,02 µg.mL-1 para narirutina e de 122,12 a 175,01 µg.mL-1 para hesperidina. A análise dos componentes principais foi capaz de diferenciar os sucos da tangerina de Cleopatra e dos porta-enxertos de calcário Rangpur de acordo com a maturação, bem como com os ambientes de extração. Os níveis de limonina e flavonóides foram reduzidos durante a maturação. Os níveis de limonina foram mais expressivos no início da colheita, especialmente no suco obtido com o sistema de extração FCOJ. Os níveis de hesperidina foram cerca de dez vezes mais altos que os níveis de narirutina em todos os sucos.

Palavras-chave: Limonina, suco de laranja pêra-rio, HPLC, narirutina, Hesperidina.

1 INTRODUCTION

Brazil is the largest world producer and exporter of orange juice. The Brazilian production of FCOJ (Frozen Concentrated Orange Juice) and NFC (Not From Concentrate) in the 2020/2021 harvest was 837.5 million ton and 821 million ton in the 2021/2022 harvest, with a decline of 1.89% due to climate problems (CitrusBR, 2022). For the 2022/2023 harvest it is expected an increase of 21% for the FCOJ production, reaching 1.138 million ton (Forbes, 2022).

Orange juice plays an important dietary role; it is an accessible source of vitamin C and also contains important bioactive compounds such as flavonoids, phenolic acids and carotenoids. The daily consumption of orange juice has been associated with various beneficial effects on human health, such as improvement of the immune system and intestinal function, decrease of cholesterol levels, reduction of risk factors for hypertension, and prevention of various chronic-degenerative diseases (Roza et al., 2007; Medina-Remon et al., 2013; Khan et al., 2014).

The quality of orange juice is influenced by many factors that affect its sensory and nutritional characteristics. Limonoids are the main compounds responsible for the bitter taste of citrus juices, which has a negative impact on juice quality. Limonin is the principal limonoid responsible for the bitter taste in orange juice. Limonin in orange juice depends on the level of the precursor limonoate A-ring lactone present in fruit, the pH and the presence of the enzyme limonoid D-lactone hydrolase. During juice extraction the insipid precursor is converted to bitter limonin (Hasegawa and Miyake, 1986) Limonin levels in the juice are influenced by the extraction settings, and the type of rootstock, variety and ripeness of the oranges (Kefford and Chandler, 1961; Scott, 1970).

Several methods have been developed to determine limonoid aglycones, such as limonin, in citrus juices. Solid phase extraction (SPE) with C18 cartridge has been widely used as being a simple, fast and accurate technique to extract limonin from the orange juice matrix. High Performance Liquid Chromatography (HPLC) has been the main technique employed for limonin separation, identification and quantification, even at low concentrations (Shaw and Wilson, 1984; Widmer, 1991; Abbasi et al., 2005; Pichaiyongvongdee and Haruenkit, 2009; Dagulo et al., 2010).

The phenolic composition of orange juice is also influenced by the variety and maturity of the fruit, edaphoclimatic conditions, cultivation system, post-harvest and processing conditions (Macoris et al., 2011; Zou et al., 2016; Mesquita and Monteiro,

2018). The main *Citrus* flavonoids are from the flavanone class, namely hesperidin and narirutin (Peterson et al., 2006; Gattuso et al., 2007; Khan et al., 2014). Hesperidin and narirutin, as well as other minor flavonoids, have been reported in orange juice from several varieties, including Pêra-Rio (Rapisarda et al., 1999; Leuzzi et al., Gattuso et al., 2007; Kelebek et al., 2009; Zou et al., 2016; Mesquita and Monteiro, 2018).

The aim of this work was to evaluate the influence of the rootstock, maturity and extraction settings on the limonin, hesperidin and narirutin levels of Pêra-Rio orange juice.

2 MATERIAL AND METHODS

2.1 CHEMICALS

Pure standards of limonin (98.3%, Chromadex), narirutin (>98%, Sigma Aldrich) and hesperidin (93.2%, USP) were used. Acetonitrile, methanol and ethyl acetate used were of HPLC grade, formic acid was of analytical grade, and ultrapure water was obtained from a Direct Q-3UV system (Millipore, USA). Bond Elut C18 cartridges (3 and 6 mL) (Agilent Technologies, USA) were used for SPE of limonin and flavonoids. For the physicochemical evaluation analytical grade reagents were employed.

2.2 ORANGE JUICE

Pêra-Rio oranges were cultivated in São Carlos, SP, Brazil (21º53'52" S, 47º53'37" W) in trees with Rangpur lime and Cleopatra mandarin rootstocks. Oranges from each rootstock were harvested at the beginning (July) and end (October) of the 2013 harvest. The maturity of the fruits was evaluated by soluble solids, titratable acidity and ratio.

Freshly-squeezed juice from oranges of each rootstock were obtained during the 2013 harvest using a JBT 391B extractor from JBT FoodTech in Araraquara, SP, Brazil, operating under NFC and FCOJ extraction settings. The juice (n=8) was frozen and stored at -20 °C until analysis.

2.3 LIMONIN EXTRACTION

After heating (93ºC/3 min) to release limonin, orange juice (10.0 mL) was centrifuged at 9000 rpm at 20ºC (20 min) and the supernatant was submitted to a clean-up step employing 500mg C18 3 mL cartridges (Bond Elut, Agilent Technologies) previously conditioned with acetonitrile (2 mL) and water (5 mL), and eluted with

acetonitrile (3 mL). Extracts were filtered through a 0.22 µm PTFE disks and stored at -20ºC until analysis. Extraction was performed in triplicate.

2.4 FLAVONOID EXTRACTION

Flavonoids were extracted from orange juice (4.0 mL) with an aqueous methanol solution (90% v/v) in ultrasonic bath (20 min). The juice solution was centrifuged at 9000 rpm and 20ºC (20 min), supernatant was collected and the procedure was repeated once. Supernatants were combined in a volumetric flask (10.0 mL) and 2.0 mL aliquots were dried under a N_2 flow. Extracts were reconstituted in an aqueous formic acid solution (0.1% v/v) (1.0 mL), filtered through 0.22 µm cellulose disks and stored at -20ºC until analysis. Extraction was performed in duplicate.

2.5 STANDARD SOLUTIONS

Stock solutions of limonin (1000 $\mu g.mL^{-1}$) were prepared in acetonitrile and work solutions were prepared as needed by successive dilutions. Stock solutions of narirutin (260 $\mu g.g^{-1}$) and hesperidin (220 $\mu g.g^{-1}$) were prepared in methanol and diluted in water as needed.

2.6 LIMONIN ANALYSIS

Limonin analysis was carried out in an HPLC-DAD (Shimadzu, Kyoto, Japan) using a Chromsep C18 250 mm x 4.6 mm, 5 µm column (Varian, USA). The mobile phase was water and acetonitrile with a flow rate of 1.0 $mL.min^{-1}$ in a linear gradient of 30%-45% acetonitrile in 30 min; column temperature was 30°C, injection volume was 20µL and detection at 210 nm (Shaw and Wilson, 1984; Ribeiro and Dias, 2002; Abbasi et al., 2005; Pichaiyongvongdee and Haruenkit, 2009; Dagulo et al., 2010). The HPLC-DAD method was validated according to international guidelines (Thompson and Wood, 2002; ICH, 2005; Magnusson and Örnemark, 2014). Calibration curve, linearity, limit of detection, limit of quantification, precision, and accuracy were evaluated.

Identification was performed in an HPLC-ESI-MS using an AmaZon SL ion trap mass spectrometer (Bruker, USA) in positive mode and scan from 70-2200 m/z. Operation conditions were capillary voltage of 4.5 kV, drying gas pressure 50 psi with a 10 $mL.min^{-1}$ flow, capillary temperature of 300ºC and injection volume of 3µL. The extracted ion chromatogram was acquired in a LTQ-Orbitrap mass spectrometer (Thermo

Fisher Scientific, USA) in positive mode at 471.2 m/z. Operation conditions were source voltage of 3.7 kV; drying gas pressure of 75 psi; auxiliary gas 20 arbitrary units; heater temperature of 500ºC; capillary temperature of 400ºC and injection volume of 10 µL.

Retention time, mass spectra and extracted ion chromatogram of juice extracts and limonin standard solution were compared for identification. For quantification, calibration curves were prepared in triplicate. Extracts were injected in duplicate.

2.7 FLAVONOID ANALYSIS

Flavonoid analysis was carried out in an HPLC-DAD (Acquity ARC, Waters, USA) using a BEH X-Bridge C18 column (250 x 4.6 mm, 5 µm) and guard column (20 x 4.6 mm, 5 µm). The mobile phase was aqueous formic acid solution (0.1%, v/v) and acetonitrile with a flow rate of 1.0 $mL.min^{-1}$ and gradient of 6-10% acetonitrile (0-16 min), 10-22% (16-36 min), 22-100% (36-38 min) and held for 5 min. Volume of injection was 20 µL and column temperature was 50ºC, as previously described and validated (Mesquita and Monteiro, 2018).

Identification was performed by comparing retention time and UV/Vis spectra of juice extracts and standard solutions of narirutin and hesperidin. For quantification purposes, calibration curves were prepared in triplicate. Extracts were injected in triplicate.

2.8 PHYSICOCHEMICAL CHARACTERISTICS

Soluble solids, ascorbic acid, total sugars and titratable acidity were determined (AOAC, 2011). Soluble solids/titratable acidity ratio was calculated.

2.9 DATA ANALYSIS

Principal component analysis (PCA) was carried out in STATISTICA 10.0 using limonin, narirutin and hesperidin levels, and physicochemical characteristics of orange juice from Rangpur lime and Cleopatra mandarin rootstocks obtained with FCOJ and NFC extraction settings during the 2013 harvest.

3 RESULTS AND DISCUSSION

3.1 LIMONIN ANALYSIS

The LC method to determine limonin in orange juice was developed based on the

methods described in literature, with some modifications (Shaw and Wilson, 1984; Ribeiro and Dias, 2002; Pichaiyongvongdee and Haruenkit, 2009; Dagulo et al., 2010). At first juice extracts were analyzed in the HPLC-DAD system in isocratic mode using acetonitrile:water (45:55, v/v) and a flow rate of 1.0 $mL.min^{-1}$. Enlarged peaks and shoulders were observed under these conditions, requiring the use of gradient mode. After testing several conditions, the final working condition was a linear gradient from 30 to 45% acetonitrile in 30 min. Also, temperature oscillations resulted in lack of resolution and repeatability of peak area values in the chromatograms. The column was then put in an oven at 30ºC.

A typical chromatogram of an orange juice extract is shown in Fig. 1a; the peak at 26.8 min was identified as limonin, which showed the same retention time of the limonin standard solution (Fig. 1b). The identity of limonin was confirmed by the mass spectrum of the peak at 26.8 min, which matched the mass spectrum of the peak of the limonin standard (Fig. 1c). The extracted ion chromatogram for m/z 471.2 (positive ion mode) of the orange juice extracts also confirmed the peak at 26.8 min to be limonin (Fig. 1d).

Validation was carried out according to international guidelines (Thompson and Wood, 2002; ICH, 2005; Magnusson and Örnemark, 2014). Calibration curves were prepared using limonin standard solutions from 0.4 to 61.5 $\mu g.mL^{-1}$. Linear regression equations with $r \geq 0.999$ were obtained, as well as the area/concentration ratio versus concentration log plots, which showed that all concentrations employed in the calibration curves were within the 95% confidence interval. Precision was evaluated using repeatability (n=3) and intermediate precision (n=9) at three concentration levels corresponding to the calibration curve (low, medium and high). RSD values from repeatability and intermediate precision assays were ≤4.5%, which indicated good precision. The limit of detection was calculated as 0.144 $\mu g.mL^{-1}$.(ICH, 2005). Accuracy was evaluated using the recovery assay. Limonin standard solutions were added to the orange juice matrix at three concentration levels (0.363, 9.09 and 15.1 $\mu g.mL^{-1}$) and recovery was expressed as the percentage of limonin obtained relative to those spiked into the matrix. Recovery was 92.6-100.4%; with a maximum RSD of 5.6%, indicating good accuracy even at a low concentration level. The limit of quantification corresponded to the lowest amount of limonin added to the orange juice matrix in the recovery assay, which was 0.363 µg.

Once validated, the method was applied to the extracts obtained from Cleopatra mandarin and Rangpur lime Pêra-Rio orange juices from the 2013 harvest. The levels of limonin in the juices ranged from 0.86 to 3.94 $\mu g.mL^{-1}$. Levels were higher at the beginning of the harvest and decreased during maturation in orange juices from the Cleopatra mandarin and Rangpur lime rootstocks. The FCOJ extraction setting showed higher levels of limonin when compared to the NFC setting in juices from both Cleopatra mandarin and Rangpur lime rootstocks at the end of the harvest, and similar levels at the beginning of the harvest. Juices from the Cleopatra mandarin rootstock showed higher levels of limonin when compared to juices from the Rangpur lime rootstock at the beginning of the harvest, and lower levels at the end of the harvest (Table 1).

The decrease in the limonin levels during the harvest naturally occurs due to the conversion of monolactones into their glycosides derivatives during the maturation of the fruit. The longer the ripeness period the more aglycones are converted into glycosides, reducing the bitter taste in the juice (Hasegawa and Miyake, 1996). The extraction setting also influences the limonin levels in orange juice; our results indicate that there was a greater reduction in the limonin levels of orange juice obtained using the NFC extraction setting. The FCOJ setting usually employs more force for the extraction and thus incorporates a higher content of albedo constituents in the juice. The type of rootstock can influence various characteristics of citrus, including the limonin levels of the juice.[7,8] No studies were found in literature regarding the limonin levels in Pêra-Rio orange juice. Orange juices from other varieties were studied by Albach et al. (1981) and the limonin contents were higher during the early season of the harvest, with a decrease at the end. Scott (1970) evaluated the effect of maturation on Murcott and Washington Navel oranges. Limonin levels increased in the juice of oranges from both varieties with different rootstocks at early maturity and then reduced.

Limonin contents usually differ according to the citrus varieties and species; levels ranged from 8.8 to 11.4 $\mu g.mL^{-1}$ in Navel fresh orange juice, 14.6 to 19.4 $\mu g.mL^{-1}$ and 2.0 to 16.9 $\mu g.mL^{-1}$ commercial Navel orange and grapefruit juices, respectively (Shaw and Wilson, 1984). Widmer (1991) reported a limonin content of 2.0 to 6.9 $\mu g.mL^{-1}$ for Valencia orange juices obtained from early and late fruits, respectively, 9.0 to 9.8 $\mu g.mL^{-1}$ from Navel, and 2.5 to 19.4 $\mu g.mL^{-1}$ and 3.5 to 7.3 $\mu g.mL^{-1}$ from White and Pink grapefruit, respectively. The limonin content of Iranian FCOJ ranged from 12.0 to 23.0 $\mu g.mL^{-1}$ (mean value 18.5 $\mu g.mL^{-1}$) and 10.0 to 22.0 $\mu g.mL^{-1}$ (mean value 17.16 $\mu g.mL^{-}$

[1]) using HPLC and UV-Vis spectroscopy, respectively.[11] Our results show that limonin levels of Pêra-Rio orange juice from both Cleopatra mandarin and Rangpur lime oranges at NFC and FCOJ extraction settings are lower than those described in literature.

3.2 FLAVONOID ANALYSIS

Narirutin and hesperidin analysis were carried out employing the conditions established by Mesquita and Monteiro (2018). The flavonoids were identified according to retention time and UV/Vis spectra of peaks from the orange juice extracts compared to pure standards. Narirutin and hesperidin were identified in the extracts of NFC and FCOJ juices from Cleopatra mandarin and Rangpur lime oranges from the 2013 harvest.

Narirutin levels ranged from 12.00 to 26.02 $\mu g.mL^{-1}$, and hesperidin levels ranged from 122.12 to 175.01 $\mu g.mL^{-1}$ amongst juices from Cleopatra mandarin and Rangpur lime oranges. Generally, levels of both flavonoids oscillated in all juices throughout the harvest. Hesperidin levels were about ten times higher than narirutin levels in all juices. Flavonoid levels decreased slightly or were about the same at the beginning and at the end of the harvest, as opposed to what was previously observed for organic and conventional Pêra-Rio orange juices (Mesquita and Monteiro, 2018). The flavonoid levels of orange juice depend on the variety and ripeness of the fruits, region and type of cultivation, as well as on processing (Bai et al., 2013; Mesquita and Monteiro, 2018; Hunlun et al., 2019). Our hesperidin levels were higher than narirutin levels in the juice from both rootstocks and extraction settings, which is in accordance to what was reported for Pêra-Rio organic and conventional orange juices[14], and Hamlin and Valencia fresh and pasteurized orange juices (Bai et al., 2013). The flavonoid levels of Mandarin, Navel and Valencia FCOJ were studied by Hunlun et al. (2013), who found that narirutin levels were higher than hesperidin levels for Mandarin juice (54.14 $\mu g.mL^{-1}$ and 24.25 $\mu g.mL^{-1}$, respectively), and about the same for Navel (45.7 $\mu g.mL^{-1}$ and 40.6 $\mu g.mL^{-1}$) and Valencia juices (39.9 $\mu g.mL^{-1}$ and 38.7 $\mu g.mL^{-1}$). It is also worth mentioning that their levels are much lower than those obtained in our study.

3.3 PHYSICOCHEMICAL CHARACTERISTICS

Physicochemical characteristics of Pêra-Rio orange juice from Cleopatra mandarin and Rangpur lime rootstocks extracted with the usual settings for FCOJ and NFC during the 2013 harvest are in Table 2. Soluble solids, total sugars and ratio of the

juices from both rootstocks and extraction settings increased during maturation, while titratable acidity decreased, as expected. Ascorbic acid also decreased during the harvest.

3.4 PRINCIPAL COMPONENT ANALYSIS (PCA)

Orange juices from both rootstocks in the NFC and FCOJ extraction settings were discriminated using PCA and represented by data in two dimensions (Fig. 2). The first two principal components (PC 1 and PC 2) explained a variation of 87.04% of the limonin, narirutin and hesperidin levels, as well as physicochemical characteristics of the juices, indicating that such characteristics are suitable to differentiate the juices from different rootstocks and extraction settings during the 2013 harvest according to their spatial location.

Juices from the beginning of the harvest were loaded negatively in PC 1 and were characterized by titrable acidity, ascorbic acid and limonin levels characteristic of oranges from early maturation, as well as higher levels of narirutin and hesperidin. Juices from the end of the harvest were loaded positively in PC 1 and characterized by higher soluble solids, total sugars and ratio, characteristic of ripe oranges. At the beginning of the harvest there is a great difference between the levels of flavonoids in juices from the Cleopatra mandarin rootstock, whereas levels are more similar in juices from the Rangpur lime rootstock. Thus, NFC and FCOJ from the Rangpur lime rootstock are closer together and NFC and FCOJ from the Cleopatra mandarin rootstock are loaded in opposite places at the beginning of the harvest and still distant at the end of the harvest.

Limonin does not have a great importance in ripe oranges, which are better characterized by the higher levels of total sugars, soluble solids and ratio. Levels decreased at the end of the harvest which consequently lowers the impact of limonin in the juice from ripe oranges in either FCOJ or NFC extraction settings.

4 CONCLUSION

Limonin, narirutin and hesperidin were identified in Pêra-Rio orange juice from Cleopatra mandarin and Rangpur lime rootstocks extracted under FCOJ and NFC settings. The developed and validated method could successfully be used to determine limonin in orange juice, showing a wide linearity, good precision and accuracy. Limonin and flavonoid levels were reduced during maturation. Juices from the beginning and end of the harvest, as well as juices obtained with FCOJ and NFC settings were differentiated.

Limonin levels are more expressive at the beginning of the harvest and especially in juice obtained with the FCOJ extraction setting. Hesperidin levels were about ten times higher than narirutin levels in all juices.

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ANEXOS

Table 1. Limonin, narirutin and hesperidin (µg.mL^{-1}) levels in Pêra-Rio orange juice from Cleopatra mandarin (CM) and Rangpur lime (RL) rootstocks, extracted with the usual setting for FCOJ and NFC, at the beginning (July) and end (October) of the 2013 harvest.

Juice	Limonin (µg.mL^{-1})		Narirutin (µg.mL^{-1})		Hesperidin (µg.mL^{-1})	
	July	October	July	October	July	October
CM NFC	3.93±0.11	0.86±0.01	12.00±1.87	15.34±3.01	137.40±25.37	122.12±3.38
CM FCOJ	3.94±0.12	1.78±0.01	26.02±0.23	15.48±0.26	244.73±31.53	161.95±10.40
RL NFC	2.13±0.01	0.90±0.03	14.38±0.17	12.24±0.57	175.01±0.10	139.13±2.79
RL FCOJ	2.30±0.04	2.25±0.05	18.77±1.13	15.05±0.69	137.78±7.07	145.23±25.17

Mean of triplicates ± standard deviation (SD).

Table 2. Physicochemical characteristics of Pêra-Rio orange juice from Cleopatra mandarin (CM) and Rangpur lime (RL) rootstocks extracted with the usual settings for FCOJ and NFC from the beginning (July) and end (October) of the 2013 harvest.

Juice	Soluble solids (ºBrix)		Titratable acidity (g citric acid/100 mL)		Ratio		Total sugars (g glucose/100 mL)		Ascorbic acid (mg/100 mL)	
	July	October	July	October	July	October	July	October	July	October
CM NFC	8.75±0.00	11.42±0.14	0.81±0.01	0.66±0.01	10.86	17.43	8.15±0.07	10.14±0.14	39.38±0.00	26.88±0.18
CM FCOJ	9.00±0.00	11.00±0.00	0.80±0.01	0.67±0.00	11.27	16.37	8.71±0.14	9.13±0.07	37.19±0.00	29.09±0.18
RL NFC	9.08±0.14	12.17±0.14	0.82±0.01	0.67±0.01	11.05	18.12	9.57±0.07	10.33±0.09	38.77±0.21	32.03±0.18
RL FCOJ	9.25±0.00	11.75±0.00	0.83±0.01	0.64±0.01	11.20	18.26	9.16±0.06	9.74±0.13	38.40±0.21	31.61±0.18

Mean of triplicates ± standard deviation (SD).

Figure 1. Typical chromatograms of (a) orange juice extract and (b) standard of limonin. (c) Mass spectrum ESI(+)-MS of orange juice extract and of limonin standard, and (d) extracted ion chromatogram in positive ion mode for *m/z* 471.2 from orange juice extract. Conditions: 30-45% acetonitrile in 30 min, 30°C, 1 mL.min^{-1}, 20µL, 210 nm (a and b).

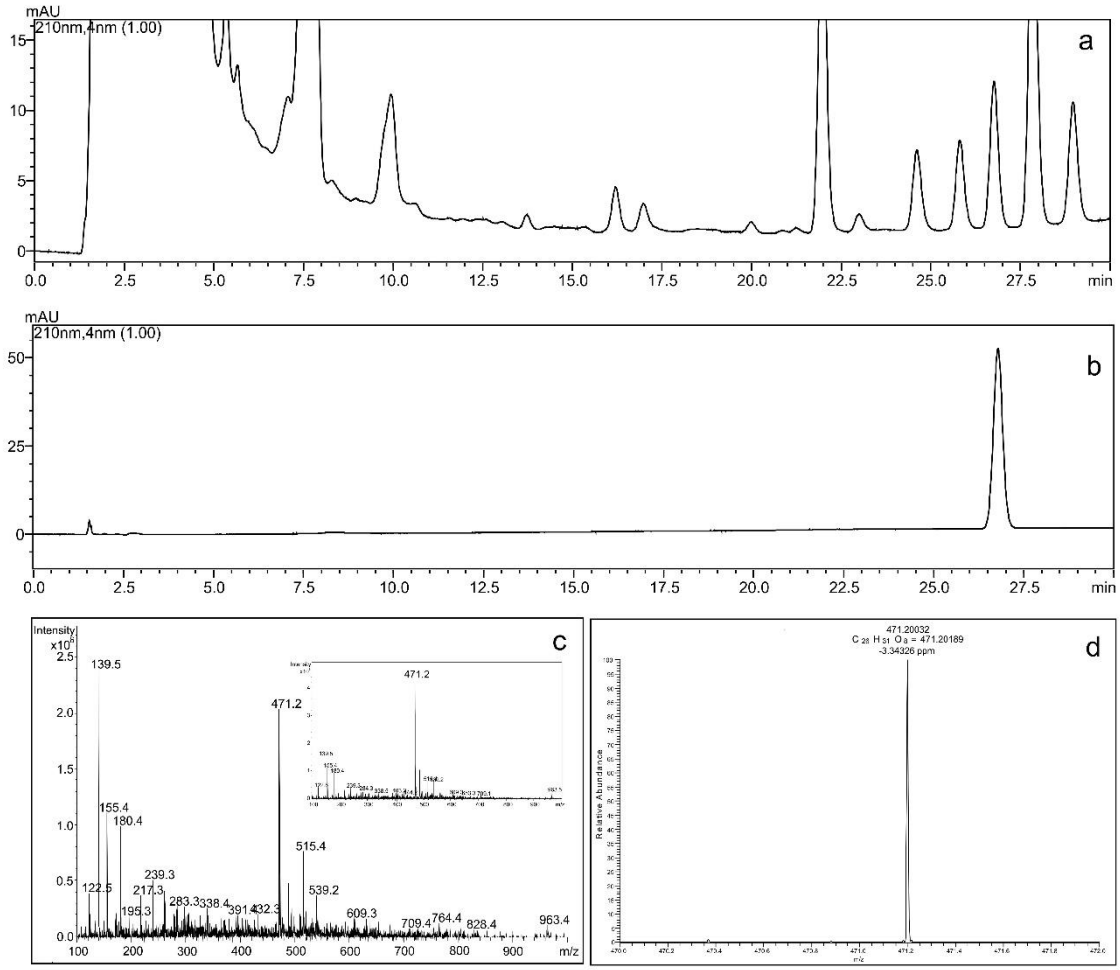

Figure 2. Principal component analysis of Pêra-Rio orange juice from Cleopatra mandarin (CL) and Rangpur lime (RL) rootstocks, extracted in the FCOJ and NFC settings at the beginning (1) and end (2) of the 2013 harvest.