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Analytical Methods

# Comprehensive analysis of phenolics compounds in citrus fruits peels by UPLC-PDA and UPLC-Q/TOF MS using a fused-core column

Vitor L. Sanches [a], Tanize A. Cunha [b], Juliane Viganó [a], Leonardo M. de Souza Mesquita [a], Lúcia Helena Faccioli [b], Marcia Cristina Breitkreitz [c], Maurício A. Rostagno [a,*]

[a] Multidisciplinary Laboratory of Food and Health (LabMAS), School of Applied Sciences (FCA), University of Campinas (UNICAMP), Rua Pedro Zaccaria, 1300, 12383-250 Limeira, SP, Brazil
[b] Department of Clinical Analysis, Toxicology and Food Science, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Av. do Café, s/n, Ribeirão Preto, SP 14040-903, Brazil
[c] Laboratory of Pharmaceutial Research and Chemometrics (LabFarQui), Institute of Chemistry, University of Campinas (UNICAMP), Rua Josué de Castro s/n, 13083-970, Campinas, São Paulo, Brazil

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ABSTRACT

In this work, a method based on ultra-high-performance liquid chromatography with a photodiode array detector (UPLC-PDA) was developed to comprehensively analyze phenolic compounds in peels of lime (*Citrus* × *latifolia*), lemon (*Citrus limon*), and rangpur lime (*Citrus* × *limonia*). The reverse-phase separation was achieved with a C18 fused-core column packed with the smallest particles commercially available (1.3 um). The method was successfully coupled with high-resolution mass spectrometry (HRMS), allowing the detection of 24 phenolic compounds and five limonoids in several other citrus peels species: key lime, orange and sweet orange, tangerine, and tangerine ponkan, proving the suitability for comprehensive analysis in citrus peel matrices. Additionally, the developed method was validated according to the Food and drug administration (FDA) and National Institute of Metrology Quality and Technology (INMETRO) criteria, demonstrating specificity, linearity, accuracy, and precision according to these guidelines. System suitability parameters such as resolution, tailoring, plate count were also verified.

## 1. Introduction

The *Citrus* genus, characterized by species such as lemon/limes, oranges, and mandarins, are of high commercial interest, comprising the most produced and commercialized fruits worldwide, with a global production forecast of 98 million tons for 2021 (USDA, 2021). It is estimated that one-third of the fresh citrus fruits produced are destined for juice manufacturing (Singh et al., 2020), and tons of peels are discarded as waste in this process. Citrus peels represent an inexpensive and environment-friendly platform for producing novel food ingredients and nutraceuticals (Rafiq et al., 2018; Forster-Carneiro, Berni, Dorileo, & Rostagno, 2013). Several products can be recovered from citrus peels, including essential oils, pectin, and phenolic compounds. They are considered a promising source of phenolic compounds, mainly those belonging to the subgroups of flavonols, flavanones, and flavones (de la Rosa et al., 2018; Rafiq et al., 2018).

The interest in phenolic compounds is related to their potential applications in health and disease prevention, such as cardiovascular diseases, cancer, and other illnesses caused by oxidative stress (Rho et al., 2011; Sibhatu et al., 2021; Thi Tam et al., 2021), among other several applications. Recently, phenolic compounds in citrus fruits, like diosmetin and quercetin, have been identified as potential therapeutic agents for coronavirus disease that emerged in 2019 (COVID-19) (Khan et al., 2021).

Unfortunately, the phenolic composition of these low-cost and highly available raw materials is variable and depends on several factors. Phenolic compounds are stress-induced phenylpropanoids (Dixon & Paiva, 1995; Zandalinas et al., 2017), and therefore, predicting their concentration is challenging. Furthermore, the variable chemical profile of the phenolics implies the need for different methods for each sample type since other compounds can be present.

Among analytical methods used to identify the profile of phenolic compounds, liquid chromatography and capillary electrophoresis coupled with spectrophotometry, electrochemical, and mass

* Corresponding author.
*E-mail address:* mauricio.rostagno@fca.unicamp.br (M.A. Rostagno).

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spectrometry detectors are the most used (Hu et al., 2017). Capillary electrophoresis has the advantages of speed, high separation efficiency, and low cost; however, it lacks stability (Hu et al., 2017). Some papers describe the use of near-infrared (NIR) to analyze phenolic compounds in food matrices; however, there is much to be improved in quantifying and identifying these species (Ferrer-Gallego et al., 2011). Furthermore, NIR has much higher detection limits than the chromatographic techniques in general.

Ultra-high-performance liquid chromatography (UPLC) is a powerful technique to analyze phenolics in a wide range of samples (Gai et al., 2021; Ahmad et al., 2021; Longo et al., 2021). UPLC systems can handle pressures of 15,000 psi or higher, allowing using columns packed with sub-2 μm particles, which, combined with small dead volumes, increases the efficiency of the chromatographic separation and peak capacity (Sanjay Shesha et al., 2021). The more efficient separation also leads to higher sensitivity, shorter analysis time, and lower solvent consumption when compared to conventional high-performance liquid chromatography (HPLC) separations.

UPLC coupled with photodiode array (PDA) is a sensitive and low-cost technique that allows exploring phenolic compounds in complex matrices. Nevertheless, since the Ultraviolet–visible (UV–Vis) spectra of phenolic compounds are very similar, another technique is necessary to identify them more accurately. In fact, UPLC combined with high-resolution mass spectrometry (HRMS) has become the most widespread approach when detailed information about the phenolic compounds present in complex matrices is required, opening a perspective of developing faster analytical methods and enabling the accurate identification of these molecules. The coupling of UPLC with high-resolution mass spectrometry (HRMS) has allowed studying phenolic compounds in complex matrices, enabling these molecules to be identified - from their mass data with higher accuracy, typically below five ppm – and opening a perspective of developing faster analytical methods for these purposes (Lucci et al., 2017). Since phenolic compounds are present in several food matrices at low concentration levels, UPLC-HRMS has been reported as an essential technique. It is used mainly with negative electrospray ionization (ESI) and quadrupole-time of flight (Q-TOF) mass analyzer (Mashitoa et al., 2021; Zihad et al., 2021). Furthermore, the widespread chemical diversity and concentration levels of phenolic compounds in food matrices make the UPLC-HRMS a valuable technique for many applications.

UPLC separation of specific classes of phenolics from citrus peels can be achieved in a few minutes (Guo et al., 2021). The usual approach is the development of specific methods for each citrus type since the composition of the samples is variable. However, it would not be necessary if mass spectrometry equipment is used, in which a specific compound tracking can be carried out by ion monitoring/recording or multiple reaction monitoring.

On the other hand, as the complexity of the sample and the number of target compounds increases, the time needed follows proportionally. Thus, despite the high efficiency of UPLC separations, an extended analysis time (sometimes hour-long methods) is still required for a comprehensive high-resolution analysis, even for only one type of sample (Wen et al., 2021).

With the development of fused-core particles, smaller particles and relatively high flow rates can be used without exceeding pressure limits, offering a balance between speed and resolution. Fused-core particles are composed of a porous shell fused to a solid core particle, which promotes a fast mass transfer of the solute inside the particles while providing a small diffusion path into (and out) of the stationary phase, resulting in faster analyses compared to the conventional stationary phases. The flatter Van Deemter curve also allows increasing mobile phase flow rate to achieve more rapid separation without sacrificing efficiency (Rostagno et al., 2011). Furthermore, extremely small fused-core particles (1.3 μm) are commercially available nowadays, providing the necessary performance and additional benefits for chromatographic separations.

In this context, the objective of this work was to develop a UPLC-PDA method for the comprehensive and fast analysis of phenolic compounds from six different citrus peels samples, exploring the higher pressure limits and low dead volume of the UPLC systems along with the benefits of a fused-core column.

## 2. Materials and methods

### 2.1. Reagents

HPLC-grade acetonitrile and methanol were provided by J.T. Baker (Phillipsburg, NJ, USA). Ultra-pure water was supplied by an ELGA Purelab flex system (Woodridge, IL, USA). HPLC-grade acetic acid was provided by Êxodo Científica (Sumaré, SP, Brazil). Hesperidin (purity > 98.0%) was bought from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Sample preparation

Samples of lime (*Citrus latifolia*), lemon (*Citrus limon*), and rangpur lime (*Citrus limonia*), key lime (*Citrus aurantiifolia*), orange and sweet orange (*Citrus sinensis*), tangerine and tangerine ponkan (*Citrus reticulada*) were purchased from a local store and washed with deionized water. The fruits were peeled, and the peel was cut into small pieces. Afterward, they were oven-dried at 60 °C in a laboratory oven (SOLAB, SL-10, Piracicaba, Brazil) for 24 h in trays (diameter of 18 cm × height of 3 cm). The samples were than ground (Oster 4126, USA) and sieved (18 mesh) before being submitted to the extraction process.

The objective of the sample preparation was to obtain a representative extract of the samples and not to carry out a quantitative study. The representative extract should contain the main compounds found in citrus peels and potential degradation products derived from drying. Oven drying is habitual since the peel is a low-value product from the industrial process. Since it is not a quantitative study, a certain degree of degradation is acceptable. If samples are processed by freeze-drying, a similar profile would be achieved.

Furthermore, this also applies to the ripening stage of the samples. Although the influence of fruit ripening is undoubtedly an interesting aspect to be discussed, Ledesma-Escobar et al. (2015), suggest that it affects the concentration of phenolic compounds but does not significantly affect the chromatographic profile of the sample. Therefore, in our opinion, considering the objective of the work to develop an analytical method, the samples and sample preparation used are adequate.

For the preparation of the extracts, one gram (1 g) of each prepared solid sample was extracted with 80 mL of methanol–water (50% v/v) on an ultrasonic bath (Elmasonic P60H, Germany) operating at 37 Hz and 150 W for one hour. Then the extracts were stored at −20 °C until analysis. The samples were filtered through a 0.22 μm nylon syringe filter (Analitica, SP, Brazil) before the analysis. The initial development of the analysis method was based on the chromatogram of the lemon peel sample since it was the most complex matrix compared to the other studied samples.

### 2.3. UPLC-PDA method development

The analysis method was developed on a UPLC-PDA system (Waters, Acquity H-Class, Milford, MA, USA) composed of a quaternary solvent manager, an autosampler manager, a column manager, and a PDA detector. The separation of the compounds in the extracts was performed on a fused core Kinetex® $C_{18}$ column (Phenomenex, 1.3 μm particle size, 50 mm length, and 2.1 mm internal diameter). Chromatograms were recorded in the 200–400 nm range, and peaks were integrated at 260 and 350 nm. Phenolic compounds usually present prominent peaks in the UV–Vis spectrum (260, 280, 325, and/or 350 nm). Most compounds absorb UV at 260 nm, considered a more generic wavelength for phenolics. Unfortunately, this wavelength is not selective, and more

compounds are detected. On the other hand, for flavonoids (the main compounds from citrus), absorption at 350 nm provides much cleaner chromatograms and is usually used for the selective detection of these compounds. Therefore, we selected 260 nm and 350 nm to record chromatograms (Lin & Harnly, 2013). Several conditions were evaluated for the development of the method, including mobile phase composition, injection volume (1–4 μL), solvent flow rate (0.4–0.5 mL/min), and column temperature (37–57 °C).

### 2.5. Identification of compounds by UPLC-Q/TOF MS

The identification of the extracted compounds was carried out on an ultra-high-performance liquid chromatography (UPLC) system (Nexera X2, Shimadzu-Kyoto, HO, Japan) coupled to a TripleTOF5600 + mass spectrometer (Sciex-Foster, CA, USA) equipped with a Turbo-V Ion Spray. The analysis was carried out with the same conditions as the optimized method (see section 3.1.). MS conditions used were: capillary voltage 4500 V in negative ion mode; nebulizer pressure 55 psi; drying gas pressure 50 psi; gas temperature 550 °C; curtain gas 35 (arbitrary units); and declustering potential 80 V. Time-of-flight mass spectrometry (TOF MS) and information-dependent acquisition (IDA) methods were used simultaneously to record the MS and MS/MS spectra. This method comprises a sweep of a mass range between $m/z$ 50 to 1200, and the generation of product ions (PI) spectra for the eight most intense ions collected. The collision energy was set at 35 V with a collision energy (CE) spread of ± 20 V. Therefore, both low and high energy fragments were recorded in a single spectrum. The PI spectra were obtained using high sensitivity mode and dynamic background subtraction. Mass calibration of the instrument was carried out using an automated calibrant delivery system (CDS) every five injections, using a negative ion calibration solution mode (AB SCIEX, Concord, ON, Canada).

### 2.4. Validation of the optimized UPLC-PDA method

Method validation was performed according to the guidelines provided by Food and drug administration (FDA) and National Institute of Metrology Quality and Technology (INMETRO) agencies. Sensitivity, limits of detection and quantification, linearity, accuracy, reproducibility, and robustness aspects were evaluated (Wells & Dantus, 2004). These parameters focused on the three more representative peaks in the citrus peel of lemon, lime, and rangpur lime samples at 260 nm. These compounds were quantified using hesperidin as a reference, the molecule for which the method was validated. It is cheaper and more readily available than other phenolics found in the samples.

System suitability was used to monitor the main chromatographic parameters (Empower 3.0 – Waters®), namely resolution, tailing factor, and theoretical plate number, which according to the USA Pharmacopoeia, must be higher than 1.5, lower than 2.5, and higher than 500, respectively (Agrawal et al., 2020; Chorilli et al., 2011). These parameters were calculated by using Empower® 3.0 software. The selectivity of the UPLC method developed for the different matrices studied was evaluated by comparing the retention time of compounds with the same UV–Vis spectrum profile in the samples.

The calibration curve of hesperidin was built by injecting the following different concentration levels of the standard: 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39 mg/L). The standard concentrations versus the area of the peaks were used to plot the calibration curve, which was statistically evaluated by the linear correlation coefficient (r), analysis of variance (ANOVA), and Cochran test to verify homoscedasticity of the residuals.

The LOD and LOQ describe the lowest concentrations for detection and quantification of the compounds, respectively. These parameters were calculated by using the following equations:

$$LOD = (SD \times 3.3)/CS \quad (1)$$

$$LOQ = (SD \times 10)/CS \quad (2)$$

where for each compound studied, SD is the estimated standard deviation of ten injections of the blank, and CS is the slope of the calibration curve.

Accuracy was evaluated by performing recovery experiments, where known concentrations of hesperidin standard prepared at three levels (8, 25, and 50 mg/L – low, medium, and high concentration) were spiked into the extract of the peels. The recovery (%) of each sample was determined as follows:

$$Recovery(\%) = (CF/OC)/AC \times 100 \quad (3)$$

where CF, OC, and AC are the concentration found by the calibration curve, original concentration, and concentration of the added solution, respectively.

The repeatability (intra-day) and intermediate precision (inter-day) of the analytical method were assessed by the relative standard deviation (RSD) of the hesperidin concentration of three repeated injections for three levels of concentrations 8, 25, and 50 mg/L in three different days. The RSD (relative standard deviation) lower than 3% was expected as the threshold to validate this parameter.

Minor changes in the method conditions can investigate aspects of robustness, which was assessed by comparing the areas of the peaks, obtained with different column temperatures (changed from 47 to 48 °C) and mobile phase flow rate (changed from 0.5 to 0.48 mL/min). The significance of the changes was evaluated by performing a *t*-test analysis (α = 95%).

## 3. Results and discussion

### 3.1. Optimization of method conditions

The method development strategy was based on determining the suitable conditions for UPLC-DAD and UPLC-HRMS to achieve a high-resolution separation in the shortest time possible for a wide range of citrus samples with different chemical profiles. A relatively short column (50 mm) with narrow i.d. (2.1 mm) with a $C_{18}$ stationary phase consisting of the smallest fused-core particle commercially available (1.3 μm) was selected, providing an extreme peak capacity for separating very complex samples. Despite the small particle size and short column, the fused-core particles allowed a relatively high flow rate for UPLC due to the lower backpressure to reduce analysis time while maintaining chromatographic performance.

The method conditions were optimized by evaluating the chromatographic separation efficiency using different solvent compositions, column temperatures, and flow rates to achieve the highest resolution in the shortest time possible.

The selected solvents for the mobile phase were water, acetonitrile, and acetic acid. When mixed with water, acetonitrile has a lower viscosity than methanol for reversed-phase separation, generating lower column backpressure. Combined with a relatively high column temperature (37–57 °C), it allows higher mobile phase flow rates (0.4–0.5 mL/min) to be used. The higher flow rates, in turn, enable exploring the flatter Van Deemter curves of fused-core particles to maintain chromatographic performance while reducing run time. Acetic acid was selected as the mobile phase modifier (0.1% in both acetonitrile and water, pH 4.7 and 3.3, respectively) due to its compatibility with UPLC-HRMS.

Initially, a gradient was established with a flow rate of 0.4 mL/min and a column temperature of 37 °C, which allowed to achieve relatively good separation of compounds of the most complex sample (lemon). Increasing column temperature from 37 to 47 °C provided narrower and more symmetric peaks and lowered the retention time of separated compounds. However, no further improvement was observed when further increasing the temperature (Fig. S1). This adverse effect may be

related to an interaction of the impact of the mobile-phase composition in the separation. As retention time of the compounds decreases due to the higher column temperature, they elute with a weaker mobile phase, which in turn promotes the diffusion of compounds inside the column, leading to broader peaks. This effect suggests that it may be necessary to adjust the gradient to compensate for the lower retention time of compounds to improve the chromatographic performance and avoid the coelution of some compounds.

Increasing flow rate from 0.40 mL/min to 0.45 and 0.5 mL/min while proportionally reducing the gradient times reduced retention time and peak width of all compounds while maintaining the separation (Fig. S2). Interestingly, it was observed that increasing flow rate affected the elution order of some of the highly retained compounds (Figs. S2 and S3). At 0.40 mL/min, deacetylnomilin **(peak 32)** eluted after limonin **(peak 33)**, while they coelute at 0.45 mL/min. At 0.50 mL/min, limonin elutes before deacetylnomilin. This effect may be explained by differences in the efficiency of the column to retain the compounds as the mobile phase linear speed increases, prompting the elution order change.

In our opinion, the changes in elution order are caused by differences in the column efficiency for these compounds with increasing flow rates. Each compound will have a different Van deemter curve, which is affected by terms A, B and C. As flow rate (linear mobile phase velocity) increases, the Van deemeter curve tends to increase (reducing the column efficiency), primarily due to the C term. Albeit lower than conventional porous particles, a higher flow rate can reduce efficiency, which may not be the same for all compounds. If one of the mentioned compounds is less retained by the column while the other is not affected as much, it could explain the differences in elution order. This behavior can be observed in a previous report (Gritti & Guiochon, 2013).

Furthermore, if co-elution were an issue, other compounds would have been detected in the MS analysis where these compounds elute. Peak purity analysis based on spectral information also did not indicate contamination of these peaks. Although we cannot eliminate the possibility of other peaks co-eluting, in our opinion, it is much more plausible that a higher flow rate is affecting the resistance to mass transfer between mobile and stationary phases of these compounds differently. The concentration of the contaminants will be low to influence to this extent the area generated

On the other hand, these compounds are not detected at 350 nm due to their UV absorption spectra. Their maximum absorption is at ~ 260–280 nm (allowing them to be detected at 260 nm). As wavelength increases, absorption decreases, and they have a reduced absorption at 350 nm, thus preventing them from being seen at 350 nm.

Different injection volumes were also evaluated to adjust the intensity of the peaks for various compounds, considering that concentration is variable and might change in new samples since phenolics are stress-induced phenylpropanoids. When the new sample presents a lower concentration of some of the target compounds, it might be possible to inject up to 4 uL without significantly impacting the method's performance (Fig. S4).

Increasing the injection volume increased the area of compounds proportionally. Increasing the injection volume from 1 uL to 2, 3, and 4 uL increased the hesperidin area from 109,704.9 ± 1,248.8 to 222,455.3 ± 107.5, 332,278.8 ± 227.7 and 442,633.3 ± 215.1, respectively. These injections resulted in concentrations of 54.02 ± 0.22 mg/L (1 μL), 109.62 ± 0.02 mg/L (2 μL), 163.63 ± 0.04 mg/L (3 μL) and 215.15 ± 0.01 mg/L using the validated calibration curve. A similar trend was observable for most compounds found in the sample. These are excellent results and reveal a potential tool for analyzing such complex samples.

However, the increased volume can impact the signal generated by compounds in higher concentrations and bring analytical problems to the analysis. When exploring higher sample volumes, it is essential to check if the signal is still in the linear range of the calibration curve. Suppose the increase in the concentration exceeds the calibration curve's linear range. In that case, it will be necessary to use a lower injection volume or make two separate injections (one using higher volume to detect compounds in lower concentrations and another low volume injection for analyzing higher concentration compounds, such as hesperidin).

In the case of hesperidin, the concentration with injections volumes of 2 μL or higher exceeded the calibration curve's validated linear range (0.39–100 mg/L). Although the validated range of the calibration curve is lower than the samples with ≥ 2 μL, the hesperiding area from injecting larger volumes remained directly proportional, suggesting a robust performance of the method even with samples with high concentrations. It is also critical to highlight no significant impact on the method's chromatogram performance (especially resolution) was observable up to 4 μL. Therefore, the injection volume can solve analytical problems detecting some compounds in low concentrations. Still, careful observation of the concentration of target analytes and the linear range of the calibration curve is required for successful application.

After minor adjustments of the gradient to improve the separation of a few overlapping compounds, a mixture of lemon, lime, and rangpur peel extracts was used to produce a complex and challenging sample. The use of a complex mix of compounds from different sources is critical since the chemical profiles of the samples can be very different due to the natural variability in the composition of the citrus peels. The method was established as follows: compounds in the extracts were separated using a fused core Kinetex® C18 column with 1.3 μm particle size, 50 mm length, and 2.1 mm internal diameter, water (A) and acetonitrile (B) both containing 0.1% acetic acid, were used as the mobile phase in gradient elution mode B (%): 3% for 0–3 min, 3–6 % for 3–4 min, 6% for 4–5 min, 6–10% for 5–6 min, 10% for 6–10 min, 10–13% for 10–10.5 min, 13% for 10.5–14 min, 13–15% for 14–16 min, 15% for 16–17 min, 15–20% for 17–18 min, 20–25% for 18–19 min, 25% for 19–20 min, 25–30% for 20–21 min, 30% for 21–22 min, 30–3% for 22–23 min and 23–26 min for 3%. The injection volume was 3 μL, the column temperature was set at 47 °C, and the flow rate was 0.50 mL/min. PDA wavelength (λ) was selected in the range of 200–400 nm, and chromatograms were registered at 260 and 350 nm. Before the analysis, each citrus extract was filtered through a 0.22 μm Nylon.

As shown in Fig. 1, it was possible to achieve an efficient separation (USP resolution and other chromatographic parameters can be consulted in Table S1) for the 34 peaks in approximately 22 min, with an overall run time of 30 min (including column clean-up and conditioning). The 3D chromatogram fingerprint scanned from 260 and 250 nm obtained from this sample is shown in Fig. S5.

The method was successfully used to analyze different peel samples of lemon, lime, and rangpur lime individually (Fig. 2) and suitable for peels of orange, sweet orange, tangerine, and tangerine ponkan (Fig. 3) without any modification. In these last samples, the chromatogram at 350 nm was more complex than the record obtained at 260 nm, and for this reason, Fig. 3 also shows the results at 350 nm. The higher complexity of the chromatogram at 350 nm is related to the composition of the samples. These samples have high flavonoid concentration, and flavonoids present an intense absorption peak in this region, resulting in a large number of peaks. The results obtained confirm that the method can be used for a comprehensive analysis of phenolics in different citrus peel matrices.

### 3.2. Validation of the optimized method

The chromatogram obtained with the mix of the three extract samples (lemon, lime, and rangpur) was tested through the following United States Pharmacopeia (USP) parameters: resolution, tailing factor, and plate count. The results are shown in Table S1, in which it is possible to conclude that the parameters studied agree with USP recommendations (Chorilli et al., 2011); USP resolution was higher than 1.5 except for the peaks 1 (1.06) and 33 (1.39), USP tailing factor was lower than 2.5, and USP plate count was higher than 500 for all evaluated peaks.

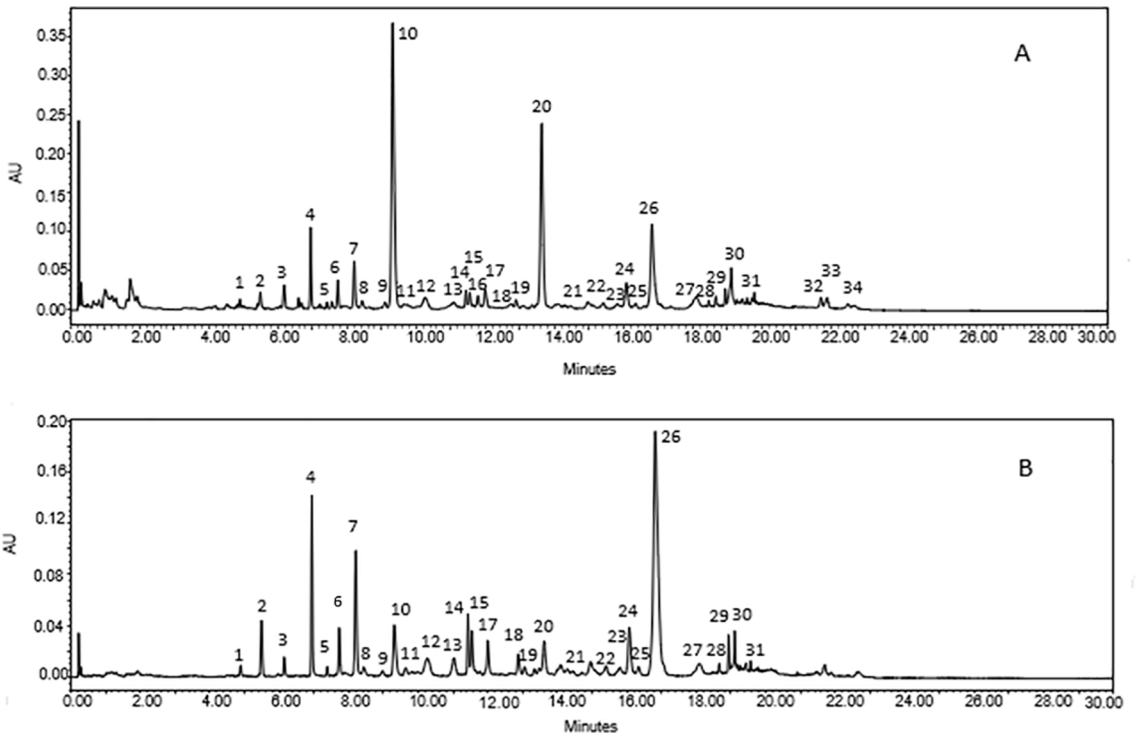


**Fig. 1.** Chromatogram representative of the sample mix of lemon, lime and rangpur citrus fruits. (A) Chromatogram recovered at 260 nm. (B) Chromatogram recovered at 350 nm.

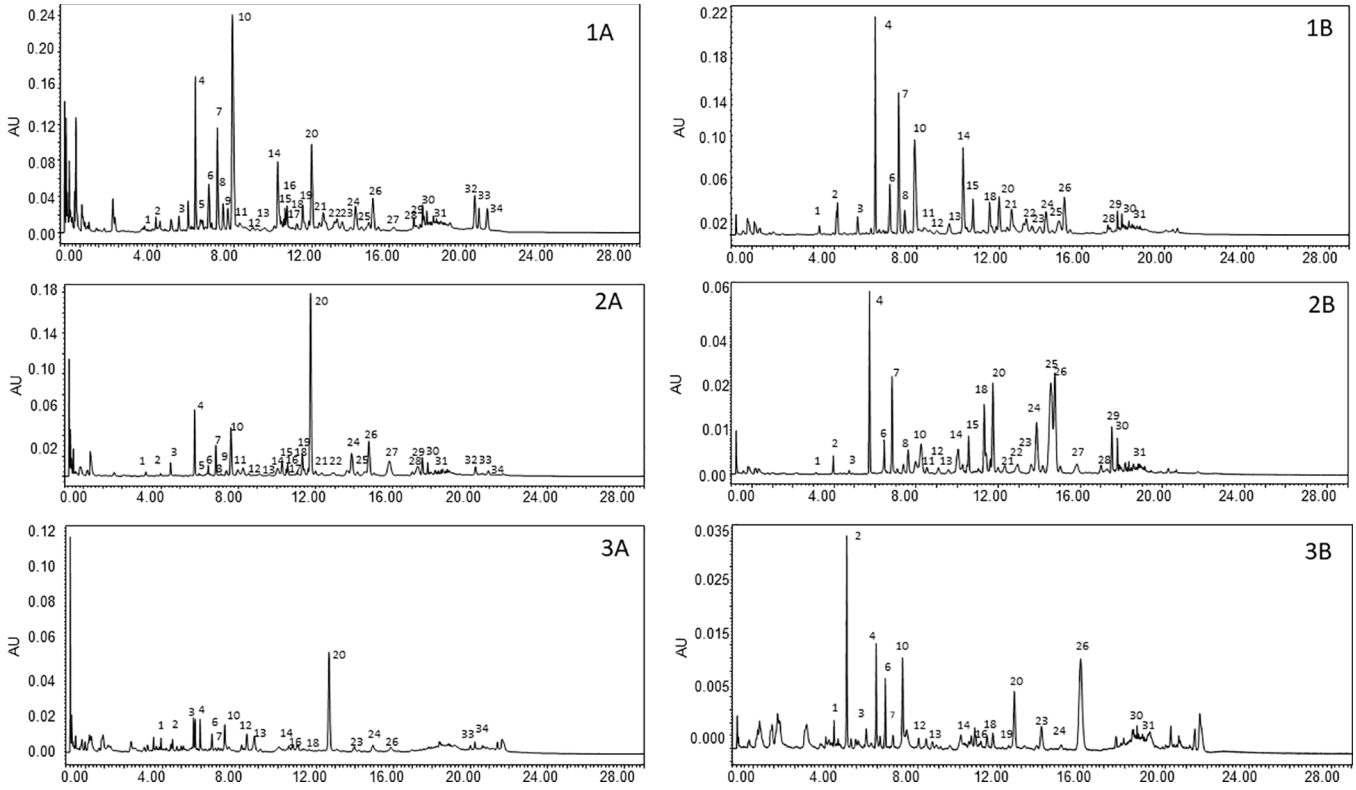


**Fig. 2.** Chromatograms obtained at 260 nm (A) and 350 nm (B) from (1) lemon, (2) lime and (3) rangpur lime.

The methods selectivity was evaluated by comparing the retention time of the most representative peaks of the three sample extracts injected individually. According to the results obtained, the method is selective for quantitative analysis of these compounds in the studied matrices since the variation between the sample peaks in the chromatogram did not exceed 0.1 min (Fig. 4).

The linearity validation was based on the correlation coefficient of hesperidin, which was equal to 0.9999. Table 1 lists the concentration

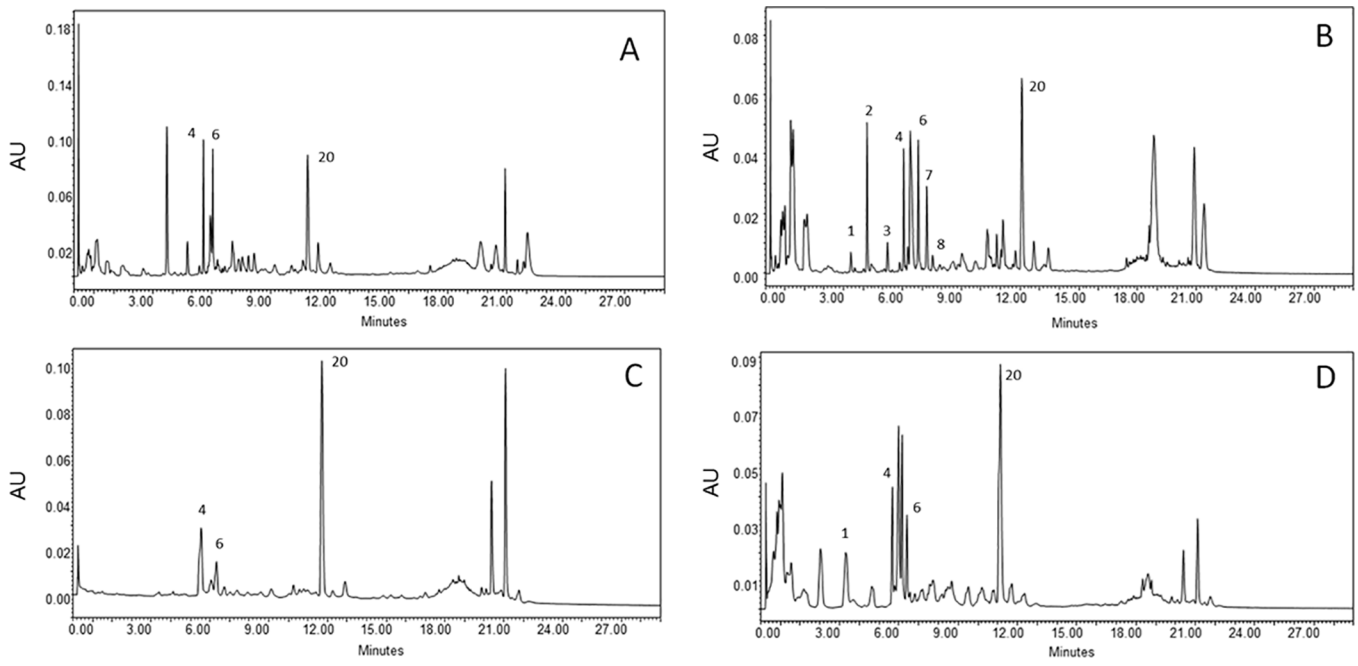


**Fig. 3.** Chromatograms obtained at 350 nm from (A) orange, (B) sweet orange, (C) tangerine ponkan and (D) tangerine.

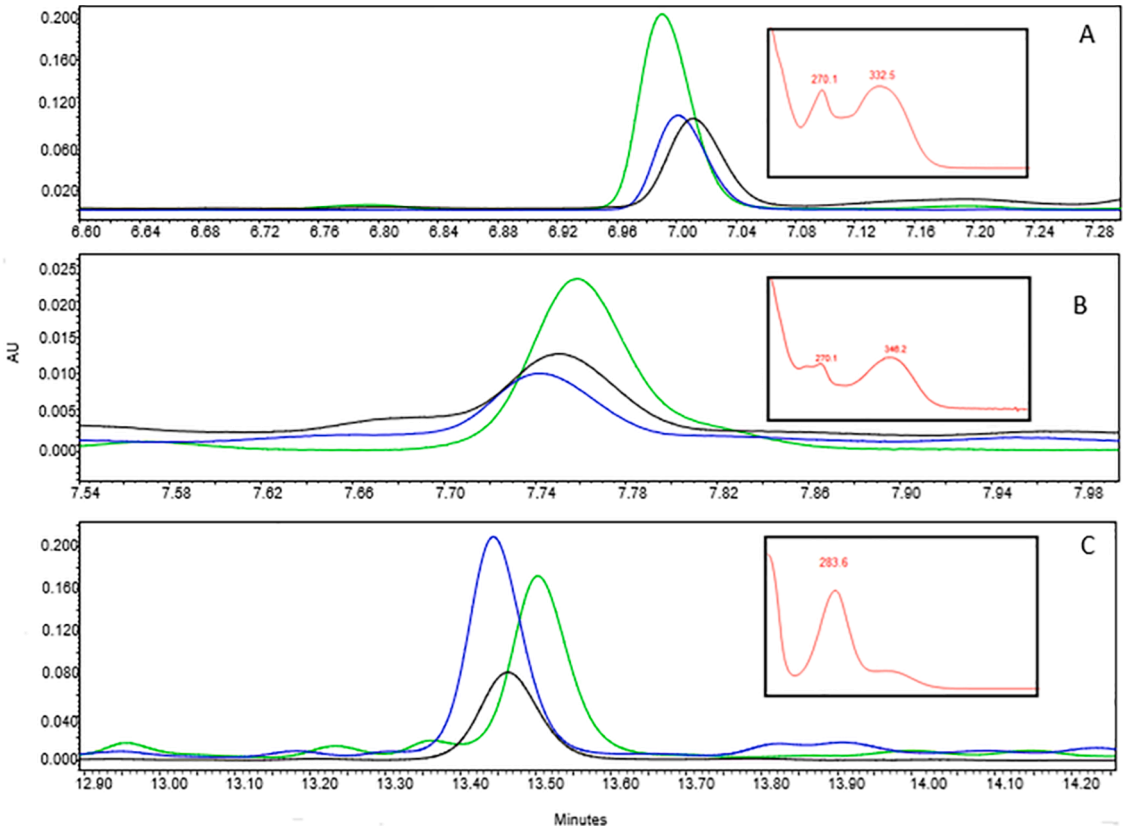


**Fig. 4.** Effect of sample matrix on the retention time in chromatograms recovered at 350 nm. (A) represents vicenin-2, (B) stellarin and (C) hesperidin. Green, black, and blue lines represent the peels samples of lemon, lime, and rangpur lime, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

range, regression equation, correlation coefficient, slope, and intercept with confidence interval (CI) of 95% from the calibration curves and the LOD and LOQ. The ANOVA F test indicated a significant regression $F_{calculated} > F_{tabulated}$ (25309 > 5.3107). The Cochran test confirmed the homoscedasticity of the analytical curve, $C_{calculated} < C_{tabulated}$ (0.019 < 0.68). The data suggest that the developed UPLC method is reliable for detecting and quantifying the target flavonoids present in the samples by relative quantification with hesperidin.

**Table 1**
Linearity, LOD, and LOQ for relative quantification with hesperidin.

| Compound | Concentration range (ppm) | Slope (CI 95%) | Intercept (CI 95) | Correlation coefficient ($r^2$) | LOD (ppm) | LOQ (ppm) |
|---|---|---|---|---|---|---|
| Hesperidin | 0.39–100 | 4069 (40) | −1378 (490) | 0.9999 | 0.17 | 0.50 |

The developed method accuracy was validated by assessing the hesperidin recovery, which was 89–110% (Table S2), within the acceptable range for the concentrations studied (80–110%). The RSD from the inter-day test was used to assess the repeatability and ranged from 0.008 to 2.09% (Table S3), which agrees with the values suggested by specialized guidelines (Zeraik & Yariwake, 2010). A quintuplicate injection of the lemon extract was also performed to assess the system repeatability. The overlapped chromatograms can be seen in the supplementary material (Fig. S6).

Even though robustness is not a figure of merit that invalidates analytical validation, some aspects were evaluated by changing the column temperature, mobile phase flow rate, and analyzing the impact of relevant compounds' concentration. The *t*-test results show that the developed method is robust and that there are no significant changes in the concentration of target compounds in comparison to the original conditions ($p > 0.05$) (Table S3).

### 3.3. Chemical profile of the samples

Identification was carried out by comparing the elution order, peak intensity, UV–Vis spectra, and Q/TOF MS data of the compounds in the extracts (Table 2). Secondary metabolite profiling methods allow quick and detailed information on the chemical composition of complex natural extracts, such as citrus peels. In this aspect, UPLC-HRMS has become one of the analytical approaches used in this field. Due to the advantages of both techniques coupling, which combine a highly efficient separation with a high-sensitivity detection method, it is possible to achieve a reliable detection and identification of a wide-ranging of metabolites allowing a comprehensive coverage of the metabolome (Perez de Souza et al., 2021).

Therefore, the extracts of lime, lemon, and rangpur peels were analyzed to demonstrate the capabilities of this method for secondary metabolite profiling. The UPLC-Q/TOF MS metabolite profiling from the

**Table 2**
Identification attempt for compounds in citrus fruits.

| Peak | UV-Vis (nm) | Retention time (min) | Identification attempt | Phenolic class | Molecular Formula | Exact mass | $[M-H]^-$ (m/z) | Mass accuracy (ppm) | MS/MS Fragments |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 324 | 4.92 | 4-Hydroxycinnamic acid (p-coumaric acid)[a,b,c] | Phenolic acid | $C_9H_8O_3$ | 164.0478 | 163.0401 | 0.2 | 119.0513 |
| 2 | 333 | 5.54 | Dihydroferulic acid[a,b,c] | Phenolic acid | $C_{10}H_{12}O_4$ | 196.0735 | 195.0668 | 2.0 | 151.0407<br>177.0196 |
| 3 | 271, 346 | 6.20 | Diosmetin-6.8-di-C-glucoside ([Lucenin-2,4'-methyl ether ][a,b,c] | Flavanone | $C_{28}H_{32}O_{16}$ | 624.1690 | 623.1640 | 3.6 | 593.1527<br>503.1186 |
| 4 | 270, 334 | 7.00 | Apigenin 6,8-di-C-glucoside (vicenin-2)[a,b,c] | Flavanone | $C_{27}H_{30}O_{15}$ | 594.1585 | 593.1538 | 4.9 | 383.0797<br>503.1227 |
| 5 | 283 ,360 | 7.43 | Eriocitrin[a,b] | Flavanone | $C_{27}H_{32}O_{15}$ | 596.1741 | 595.1681 | 2.1 | 287.0565<br>265.1073 |
| 6 | 271, 345 | 7.74 | Chrysoeriol 6,8-di-C-glucoside (stellarin-2) [a,b,c] | Flavanone | $C_{28}H_{32}O_{16}$ | 624.1690 | 623.1636 | 3.0 | 503.1209<br>265.1084 |
| 7 | 270, 345 | 8.23 | Vitexin 2''-xyloside[a,b,c] | Flavanone | $C_{26}H_{28}O_{14}$ | 564.1471 | 563.1424 | 3.1 | 413.0866 |
| 8 | 269, 320 | 8.45 | Diosmetin 7-neohesperidoside (neodiosmin) [a,b] | Flavanone | $C_{28}H_{32}O_{15}$ | 608.1744 | 607.1677 | −0.23 | 299.0609<br>283.0273 |
| 9 | 267, 340 | 9.02 | Rhoifolin 4-glucoside[a,b] | Flavanone | $C_{33}H_{40}O_{19}$ | 740.2163 | 739.2113 | 3.0 | 431.1009<br>341.0722 |
| 10 | 283,335 | 9.35 | Neoeriocitrin[a,b,c] | Flavanone | $C_{27}H_{32}O_{15}$ | 596.1741 | 595.1675 | 1.1 | 287.0566<br>577.1558 |
| 11 | 270, 335 | 9.66 | Quercetin-3-O-neohesperidoside[a,b] | Flavanone | $C_{27}H_{30}O_{16}$ | 610.1885 | 609.1477 | 2.6 | 301.0335 |
| 12 | 269,345 | 10.29 | Luteolin-neohesperidosidose[a,b,c] | Flavanone | $C_{27}H_{30}O_{15}$ | 594.1583 | 593.1525 | 2.2 | 285.0455 |
| 14 | 271, 346 | 11.06 | Diosmetin-7-O-rutinoside diosmin[a,b,c] | Flavanone | $C_{28}H_{32}O_{15}$ | 608.1741 | 607.1695 | 2.1 | 299.0569 |
| 15 | 254, 346 | 11.44 | Kaempferol-3-O-Rutinose[a,b,] | Flavanone | $C_{27}H_{30}O_{15}$ | 594.1584 | 593.1527 | 2.5 | 285.0400<br>447.0933 |
| 16 | 267, 345 | 11.56 | Diosmetin 8-C-glucoside[a,b,c] | Flavanone | $C_{22}H_{22}O_{11}$ | 462.1162 | 461.1098 | 1.9 | 341.0692<br>371.0805 |
| 17 | 269, 346 | 12.02 | Apigenin 7-O-neohesperidoside (rhoifolin)[a,b] | Flavanone | $C_{27}H_{30}O_{14}$ | 578.1636 | 577.1581 | 3.1 | 269.0467 |
| 18 | 266, 331 | 12.91 | Isorhamnetin-3-O-neohesperidoside[a,b,c] | Flavonol | $C_{28}H_{32}O_{16}$ | 624.1678 | 623.1640 | 3.6 | 315.0532 |
| 19 | 252, 346 | 13.08 | Limocitrin-neohesperidoside[a,b,c] | Flavonol | $C_{29}H_{34}O_{17}$ | 654.1801 | 653.1750 | 4.1 | 345.0616<br>329.0310 |
| 20 | 283 | 13.53 | Hesperidin[a,b,c] | Flavanone | $C_{28}H_{34}O_{15}$ | 610.1898 | 609.1837 | 2.0 | 301.0733<br>325.0712 |
| 30 | 215, 269, 316 | 19.11 | Isosakuranetin-7-O-rutinoside[b,c] | Flavanone | $C_{28}H_{34}O_{14}$ | 594.1954 | 593.1889 | 2.2 | 285.0787<br>164.0113 |
| 31 | 215 | 19.55 | Nomilinic acid 17-O-β-D glucoside[a,b,c] | Limonoid | $C_{34}H_{48}O_{16}$ | 712.2942 | 711.2897 | 3.9 | 651.2708<br>607.2804 |
| 32 | 218 | 21.46 | Diacetylnomilin[a,b] | Limonoid | $C_{26}H_{32}O_8$ | 472.2097 | 471.2017 | −1.6 | 453.1517<br>325.1820 |
| 33 | 218 | 21.70 | Limonin[a,b,c] | Limonoid | $C_{26}H_{30}O_8$ | 470.1941 | 469.1883 | 3.2 | 451.1484 |
| 34 | 219 | 22.60 | Nomilin[a,b,c] | Limonoid | $C_{28}H_{34}O_9$ | 514.2203 | 513.2119 | −2.1 | 495.2003<br>469.1850 |

Superscribed letters indicate: a – lemon, b – lime, and c – rangpur lime. $Massaccuracy(ppm) = \frac{Measuredmass - Exactmass}{Exactmass} \times 10^6$.

citrus peels is represented in Fig. S7. An extracted ion chromatogram (XIC) was also generated, which displays the intensity of the $[M-H]^-$ ions of vicenin-2, stellarin-2, and hesperidin for lemon peel samples (Fig. S8), the phenolic compounds which the method was validated concerning selectivity and robustness. The results were processed using MS-DIAL (v. 4.7) (Tsugawa et al., 2015).

Twenty-four phenolic compounds and 5 limonoids (terpenoids) were identified in the citrus peels' extracts. For the identification of compounds, the accurate single MS mass of parent ion and respective MS/MS fragments of each one was compared with those contained in online chemical databases, such as food database (FooDb) and mass bank and those previously reported in citrus fruit (Avula et al., 2016; Brito et al., 2014; Ledesma-Escobar et al., 2015; Buyukkurt et al., 2019; Wen et al., 2021).

Data corresponding to the detected compounds is summarized in Table 2 and Table S4. As can be seen in this table, three different subclasses of phenolic compounds and five limonoids (terpenoids) were found in the samples. According to the FDA agency recommendations, only compounds whose $m/z$ of the fragment $[M-H]^-$ presented mass accuracy of $\leq 5$ ppm should be considered (FDA, 2015), which implies tolerance of mass variation up to the third decimal place. Additionally, the identification of some ions was confirmed with the injection of analytical standards under the same analysis conditions.

Flavones and flavanones were the most prevalent subclass of compounds identified in the citrus peels. Among them, vicenin-2, stellarin-2, and hesperidin were some of the most abundant compounds found. Because of the important biological activities that these molecules present, such as lung and colon antitumor, neuroprotective for Alzheimer disease, and analgesic (Guazelli et al., 2021; Hamdan et al., 2020; Mathpal et al., 2021), they are of particular relevance to researchers in the area.

### 3.4. Comparison to the existing methods

Several articles were published on citrus fruits matrices recently, such the study by Coelho et al. (2021). In this report, the authors developed an HPLC method where 17 flavonoids were separated in 33 min, using mobile phase consisted of a solution of phosphoric acid 0.1 mol/L and methanol acidified with 0.5 % phosphoric acid solution, in a Zorbax Eclipse Plus RP-C18 column (100 × 4.6 mm, 3.5 μm) a temperature set at 35 °C, whereas, the developed method allows the analysis of 22 flavonoids in 22 min. Furthermore, considering other parameters such as injection volume (20 μL versus 3 μL in the present study), mobile phase flow (0.8 mL/min versus 0.5 mL/min in the present study), and acids used in the mobile phase (phosphoric acid versus acetic acid in the present study), the developed method is a closer agreement with the principles of green chemistry (Zuin et al., 2021). It is also important to point out that the study of Coelho et al. was conducted with fresh fruit juice of citrus, a much simpler matrix than citrus peels, which contain a greater complexity of compounds such as lignin and pectin.

In another report by Wen et al. (2021), the analysis by UPLC-MS/MS of a citrus sample, the *Citrus aurantium* species (bitter orange), was able to identify 58 phenolic compounds using a ACE EXCEL 1.7 C18-PFP column, (2.1 × 100 mm, 1.7 μm, ACE, UK), mobile phase consisted of solvent $H_2O$ with 0.1% formic acid, v/v and solvent acetonitrile with 0.1% formic acid, v/v, with a flow rate of 0.4 mL/min, column temperature maintained at 35 °C and 1 μL the injection volume. However, the analysis method time used reaches 60 min, almost three times higher than the method developed in this work.

Another interesting study was presented by Guo et al. (2021), where 31 flavonoids from mandarin citrus fruit were separated in 15 min by UPLC coupled with triple quadrupole mass spectrometry (QqQ-MS/MS) using ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm, Waters) at 40 °C, fow rate of 0.3 mL.min and injection of 1.0 μL. The mobile phases was constituted of acetonitrile and water containing 0.1% formic acid. Unfortunately, only one citrus sample was analyzed, and the method is not helpful for a cheaper instrument, such as the UPLC-PDA, which is much easier adapted to routine analysis in a company's quality control laboratory compared to a mass spectrometry system.

It should also be noted that due to the complexity of phenolic compounds from citrus matrices, using an HRMS system allowed the identification of these compounds more effectively. Within this context, we reiterate the scientific contribution that our work adds since no proposals were found where a UPLC method was compatible with both PDA and HRMS systems for different types of citrus peel.

## 4. Conclusion

The developed UPLC method is compatible with both PDA and HRMS detection systems. Moreover, the method was validated regarding specificity, linearity, accuracy, and repeatability. Since 29 compounds of different classes, including phenolic compounds (phenolic acids, flavanones, flavones, flavonols) and limonoids have been identified from lemon, lime, and rangpur samples, we believe that this method enables metabolomics studies in citrus samples to be carried out in the future. The use of the fused core column enabled, in addition to good chromatographic resolution, low mobile phase volume, 15 mL per injection, where within this total volume, the organic phase used (acetonitrile) corresponds to only 13%, that is, from the perspective of solvent reduction toxic and/or harmful to the environment, the proposed method is in line with the principles of Green Chemistry. Furthermore, the method can be used as a basis for developing shorter methods for analyzing citrus peels focusing on specific compounds of interest, and for the analysis of other citrus samples (such as juices).

## CRediT authorship contribution statement

**Vitor L. Sanches:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Tanize A. Cunha:** Formal analysis, Writing – review & editing. **Juliane Viganó:** Writing – review & editing. **Leonardo M. de Souza Mesquita:** Writing – review & editing. **Lúcia Helena Faccioli:** Resources, Writing – review & editing. **Marcia Cristina Breitkreitz:** Validation, Writing – review & editing, Supervision. **Maurício A. Rostagno:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2022.100262.

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