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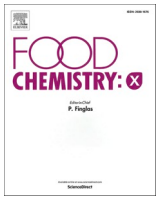

Investigation of pectin deficiency in modulating the bioflavonoid profile of orange processing waste: A sustainable valorization of industrial waste

Symone Costa de Castro [a], Júlio César Jeronimo Barbosa [b], Bruno Sozza Teixeira [a], Taicia Pacheco Fill [b], Ljubica Tasic [a,*]

[a] Laboratory of Biological Chemistry (LQB), Institute of Chemistry, Universidade Estadual de Campinas (UNICAMP), 13083-970 Campinas, SP, Brazil
[b] Laboratory of Biology Chemical Microbial (LaBioQuiMi), Institute of Chemistry, Universidade Estadual de Campinas (UNICAMP), 13083-970 Campinas, SP, Brazil

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ABSTRACT

Orange processing waste (OPW) generated by the processing of oranges, as well as other citrus fruits, is a major source of pectin in the market nowadays. The residues generated during the pectin extraction process may contain many phytochemicals, including flavonoids. We use state-of-the-art techniques such as liquid chromatography high-resolution mass spectrometry (LC-HRMS/MS) and feature-based molecular network (FBMN) to annotate the flavonoids in OPWs. In particular, four flavonoids, hesperidin, naringin, diosmin, and hesperetin were quantified in the samples by LC-TDQ-MS. In total, 32 flavonoids from different classes were annotated, of which 16 were polymethoxylated flavonoids, 13 were flavonoid glycosides and 3 were flavanone aglycones. The results showed that flavonoid glycosides remain in high concentrations in OPWs from pectin factories even after pectin extraction by harsh conditions. The results show an exciting opportunity to harness the untapped potential of pectin factory waste as a renewable source for the extraction of glycoside flavonoids.

1. Introduction

Orange processing waste (OPW) and other citrus waste, subproducts of the processing of orange fruits are becoming an environmental issue because of the large quantities produced every year. Juice production is the main source of this waste, composed of peels, seeds and pulp (Suri et al. (2022); Zema et al. (2018)). Brazil is responsible for more than 50 % of the world market of orange juice production. According to the United States Department of Agriculture, the forecast of orange juice production in Brazil in 2022/23 is 1.1 million tons (USDA, 2023). In 2016, processing industries generated around 10,929.50 metric tons of citrus waste (Kundu et al., 2020). The common fate of OPW is animal feed or landfilling waste (Castro, Soares, & Tasic 2023). However, OPW contains a variety of active phytochemicals, vitamins, dietary fiber, pectin, and antioxidants such as flavonoids that can be extracted to the best use of this waste (Sharma et al., 2022; Wedamulla, Fan, Choi, & Kim, 2022).

Some factories are currently using OPW to extract pectin, which is found in high concentrations in orange peels, about 20–30 % of the dry matter basis, and has high economic value. Pectin is extracted from citrus peels after d-limonene extraction (Ciriminna et al. (2015)). It is worth mentioning that citrus peels, which include oranges, are the main source of commercial pectin, 85.5 % of the total (Belkheiri et al., 2021). Pectin is a polysaccharide composed mainly of a linear chain of galacturonic acid units (D-GalA) linked by 1–4 glycosidic bonds and reside in the primary cell and middle lamella of plants (Baghdadi et al., 2023). The applications of citrus pectin are extensive. In the food industry, it is commonly used in the production of jams, and jellies, due to its gelling properties. Additionally, citrus pectin finds its way into beverages, as it can stabilize and enhance the texture of fruit juices and fruit-based drinks (Freitas, Coimbra, Souza, & Sousa, 2021). In industry, pectin is extracted from OPW and other sources using inorganic acids such as nitric acid and hydrochloric acid at pH 1.5–3.0 under heating techniques (60–100 °C) (Picot-Allain, Ramasawmy, & Emmambux, 2020). Pectin factories usually are located close to the source of raw material, as their processes are generally expensive. There are six main producers of pectin, heavily consolidated worldwide, Herbstreith & Fox, Naturex/Obipektin, Danisco/Dupont, Cargill, CP Kelco, Yantal Andre Pectin (Ciriminna, Fidalgo, Delisi, Ilharco, & Pagliaro, 2016). Although it represents an advance in terms of circular economy, the extraction of pectin also generates wastes in their process (pectin by-products), once there are other components in the orange cell walls, such as other fibers

* Corresponding author.
E-mail address: ljubica@unicamp.br (L. Tasic).

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(cellulose, hemicellulose), and lignin, and minor compounds, such as antioxidants. Among the bioactive molecules in orange processing waste from orange juice and maybe in pectin by-products, flavonoids deserve attention.

Flavonoids are plant secondary metabolites with a basic skeleton of 15 carbon atoms distributed in three rings called A, B and C, where the C ring contains an oxygen atom. Their subgroups include flavones, flavonols, flavanones, isoflavones, anthocyanins, flavanols, and chalcones (Shen et al., 2022). These compounds differ in the degree of unsaturation and oxidation of the C ring to which the B ring is attached. When the B-ring is attached to the C-ring at position 3, they are called isoflavones. Flavanols are those in which the B-ring is attached in position 2 of the C-ring; anthocyanins have a positive charge in the oxygen atom. Flavones and flavonols have a ketone group, and flavonols also have an OH group in the C3 position of the C ring. Finally, flavanones have a ketone group and differ from flavones by the absence of unsaturation between C2-C3 (Panche, Diwan, & Chandra, 2016; Shen et al., 2022). Flavonoids normally occur in various modified forms, which are obtained by additional hydroxylation, methylation and, one of the most important modifications, glycosylation. The glycosylation of flavonoids can occur as *O*-glycosides or *C*-glycosides, in which the sugar is attached to the flavonoid basic nucleus by C—O and C—C bonds, respectively (Cuyckens & Claeys, 2004).

Common natural sources of flavonoids include fruits, vegetables, seeds and flowers. The type of dominant class of flavonoid in each source varies. For example, flavonols are found mainly in apples, onions, potatoes, nuts, and so on, and flavones are found in broccoli, oregano, lettuce, and citrus fruits, among others (Dias, Pinto, & Silva, 2021). Even in the citrus genus, there are variations between the class of flavonoids found in each species. In oranges, the flavanones are dominant, although there is the presence of other flavonoid subgroups (Addi et al., 2022). In citrus species, flavonoids generally appear in the form of glycosides and hesperidin is dominant in oranges, about 1.2–2.8 % on a dry basis (Shen et al., 2022; Victor, David, Cortez, Leite, & Silva, 2020). The range in mg/g of peel dry basis of hesperidin in some citrus species is: 0.002 to 9.42 in lemon, 3.95 to 80.90 in mandarin, and the range of polymethoxyl flavones is 0.08 to 0.29 for sinensetin, 0.2 to 14.05 for nobiletin, 0.16 to 7.99 for tangeretin and heptamethoxyflavone for citrus peels (Addi et al., 2022). Numerous studies are showing the beneficial effects of flavonoids in human health such as the prevention of cancer, cardiovascular diseases, antibacterial infections, anti-inflammatory, and neuroprotective effects, among others due to their antioxidant effects (Maleki et al. (2019); Ciumărnean et al., 2020).

Many reports about the extraction and identification of citrus flavonoids can be found in the literature (Deng et al., 2022; Wang, Chen, Guo, Abbasi, & Liu, 2016; Deng et al., 2023). However, to the best of our knowledge, there are few reports about the identification and quantification of flavonoids in pectin by-products. For example, Zhou et al. (2022) studied the extraction of hesperidin (Hsd) after pectin extraction and found that it can greatly increase the yield of Hsd. The authors propose that selective removal of pectin may disrupt cellular architecture, exposing hesperidin crystals, which enhance mass transfer rates. In this sense, it is possible to investigate the effects of pectin absence in OPW generated by pectin factories on flavonoid profiles. Interestingly, there is a lack of documented efforts in the literature related to identifying and quantifying flavonoids produced from byproducts of industrial pectin extraction. To achieve the total flavonoid profile of different matrices, a feature-based molecular network (FBMN) combined with dual ionization mode (MS/MS) is an adequate approach to be used. This technique has advantages through classical molecular networks, once it can improve the annotation accuracy (Li et al., 2022).

Therefore, we aimed to systematically examine the impact of pectin depletion on the flavonoid composition found in OPWs and find a way to add value to this kind of industrial and agricultural residues. To achieve this goal, ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was used combined with the Feature-Based Molecular Networking FBMN approach. We aimed to fill this crucial gap in knowledge and make a substantive contribution to the scientific understanding of waste utilization in matrices rich in flavonoids.

2. Materials and methods

2.1. Materials - Reagents and samples

Standard flavonoids, hesperidin (> 97%), diosmin (analytical grade), naringin (> 95%) and hesperetin (analytical grade) were purchased from Sigma-Aldrich (St. Louis, USA). Methanol, acetonitrile (HPLC grade), *m*-hydroxydiphenyl (> 85%, 2622505G), and monohydrate D-(+)-Galacturonic acid (> 97%, 48280-25G) were acquired from Sigma-Aldrich (St. Louis, USA). Sodium tetraborate (97%), sulphuric acid (98%), and sodium hydroxide (97%) from Dinâmica (Indaiatuba, Brazil). OPW_1, OPW_2 and OPW_3 were kindly ceded by a pectin factory (Brazil) and were crushed with a blender. OPW_1 and OPW_2 are samples generated at the end of the pectin extraction process whereas OPW_3 is a by-product generated at the beginning of the process, composed basically of juice sacs. Citrus peels from orange, OPW_4, were collected from a local restaurant (Unicamp, Campinas-SP, Brazil, in November 2022) after juice extraction. In the lab, the peels were separated from the pulp and processed with a blender. All OPW samples are from the same variety of sweet orange (*C. sinensis*) cultivated in Sao Paulo, Brazil, and all were collected at maturity. These samples were stored in the refrigerator (-20 °C) until the essays. The total solids content of the samples was obtained according to the AOAC method 20.013 (AOAC, 2023). It was determined as a percentage (mean ± standard deviation, n = 3) for OPW_1 (16 ± 0.15%), OPW_2 (13 ± 0.45%), OPW_3 (10 ± 0.08%), and OPW_4 (20 ± 0.35%).

2.2. Pectin content (D-GalA measurement)

The *m*-hydroxydiphenyl colorimetric assay was used to quantify the uronic acid D-GalA in the sample cell wall, with some modifications (Ahmed & Labavitch, 1977; Benvenutti et al. (2022)). The cell wall components were dissolved in sulfuric acid as follows: approximately 5 mg of the washed sample was mixed with 2 mL of cold concentrated sulfuric acid under stirring in a water ice bath; then, 0.5 mL of distilled water was added dropwise and the mixture was stirred for 5 min for the complete dissolution of the sample. The sample was transferred to a 10 mL volumetric flask that was filled with water. 0.6 mL of the cell wall solution was mixed with 3.6 mL of chilled tetraborate reagent (0.0125 mol/L in concentrated sulfuric acid) in a test tube. The tubes were heated in a boiling water bath for 5 min. After cooling, 60 μL of the 0.15% (m/v) *m*-hydroxy diphenyl in 0.5% (m/v) NaOH solution was added and mixed. The absorbance of the mixture was read at 520 nm with a UV–Vis spectrophotometer (FEMTO - Cirrus 80, Sao Paulo, Brazil). Blanks in which 60 μL of 0.5% NaOH solution rather than *m*-hydroxydiphenyl were made to correct any color produced by neutral sugars present in the materials. The assay was performed in triplicate for each sample. A standard curve of monohydrate galacturonic acid ranging from 10.0 to 80.0 μg/mL was used for the quantification ($GalA = 108.14\ x\ x_{absorbance}$). The pectin concentration was expressed in g/100 g of the GalA equivalent.

2.3. Flavonoids extraction

To 3 g of the wet samples (OPW_1, OPW_2, OPW_3, and OPW_4) in an Erlenmeyer flask was added 15 mL of methanol and then, the mixture was heated at 55 °C for 30 min under stirring. After cooling to room temperature, this mixture was filtered. The process was repeated three times. The supernatants were combined in a volumetric flask of 50 mL, which was filled with methanol. The extracts were filtered with 0.22 μm PTFE membrane before LC injections.

2.4. UHPLC–HRMS/MS conditions

Analysis LC-HRMS/MS were performed in a UHPLC-MS/MS - Thermo Q-Exactive Orbitrap Mass Spectrometers (MS) - Dionex UltiMate 3000 RSLCnanoSystem, operating in positive and negative ionization modes. LC analyses were performed in a C18 (2.1 mm x 50 mm, 1.7 μm; Waters) column. The mobile phase used (A) water, and (B) acetonitrile - ACN with 0.1 % formic acid. The gradient was initiated with 5% of mobile phase B, which was maintained for 7 min (initial-8 min). Then phase B was set as 95% for 1 min (8–9 min) and again returned to 5% (9.1–13 min). The initial parameters were maintained at 13 to 15 min. The optimized analysis time was 15 min. Mass spectrometry analysis was performed in DDA full scan, with a scan from *m/z* 100 to 1500 Da. MS/MS fragmentation spectra were acquired from the five most intense ions per scan. The injection volume was 5 μL. The acquisition parameters follow - Spray voltage 3,500 V for positive mode and 3,200 V for negative mode; ion source temperature 300 °C; sheath gas flow 35 bar; full mass resolution 70,000 and MS/MS resolution 17,500; collision energy 20/35/45 eV (NCE mode).

2.5. Data processing

MzMine 3.9.0 software (Schmid et al., 2023) was used to process the UHPLC-HRMS/MS data; the parameters used for pre-processing the analysis of MS1 and MS2 mass spectra are presented in Table S1. The flavonoids from *Citrus sinensis* processing waste (OPWs) were identified using the GNPS database (Wang et al., 2016). The GNPS Feature-Based Molecular Networking (FBMN) analysis was performed following the protocol already established on the website (https://ccms-ucsd.github.io/GNPSDocumentation/featurebasedmolecularnetworking/). The MS/MS spectra were selected with only the top five fragmentation ions in the ± 5 ppm window. The mass tolerance of precursor ions and MS/MS fragment ions was adjusted to 0.02 Da in both cases. The spectral libraries used for this study were pre-established according to the features table generated by MZMine 3.9.0 (Katajamaa, Miettinen, & Orešič, 2006). Correspondences between network spectra and libraries were filtered to show values greater than 0.7 (Cosine Score).

The classification of flavonoids and other metabolites in this study adhered to the criteria established by Schymanski et al. in 2014: Annotation Level I, which confirms the structural identity through a direct comparison with reference standards; annotation Level II, involving the correlation of MS/MS fragments with spectra found within the GNPS database; annotation Level III, where structural evidence is based on *in silico* generated MS and MS/MS fragments; and annotation Level IV, reserved for annotated SMs where the molecular formula can be unequivocally determined using data from LC-HRMS analyses.

The simulated spectra used were generated by SIRIUS 5.7.0 (Dührkop et al., 2019) and the input data were automatically compared and classified against databases present in SIRIUS 5.7.0 (Feunang et al., 2016). Finally, all MS/MS spectra, together with their metadata in mzXML format, were deposited in an open format in the GNPS/MassIVE (Wang et al., 2020) public data repository. Finally, the area of the corresponding flavonoids annotated ions was calculated manually using the software Thermo Xcalibur 3.0.63 (Copyright 1988–2013 Thermo Fisher Scientific Inc.), and correlation graphs of the calculated areas were constructed to analyze the abundances of these metabolites in the different OPW's, using the software PRISM 8.0.1.

2.6. Flavonoids quantification by LC-MS/MS

Four standard flavonoids were used for quantification - hesperidin, diosmin, naringin and hesperetin. The stock solutions of these compounds were made as 100 mg/mL in MeOH. In the case of diosmin, MeOH with DMSO (30%, v/v) was used for proper solubilization. Calibration curve equations of these flavonoids were used for flavonoid quantification in the OPW samples. Linear ranges (n = 7) were set at concentrations between 0.250–10 μg/mL for hesperidin and naringin, and 0.015–1.0 μg/mL for diosmin and hesperetin. LODs and LOQs were calculated as 3.3 SD/S and 10 SD/S, respectively, in which SD is the standard deviation of the intercept, and S is the coefficient slope of the equation. For the separation of the compounds, a UPLC ACQUITY (Waters, Milford, MA) was used. For compound separations, an ACQUITY UPLC BEH C18 (2.1 mm × 50 mm, 1.7 μm; Waters, U.K.) column was used. The conditions were: binary mobile phase - Water (A) and acetonitrile - ACN with 0.1 of formic acid (B); gradient elution: 0–7 min, 5% B; 8–9, min 95% B; 9.1–13.5% B. The injection volume was 2 μL and the flow rate was 0.4 mL/min. A Xevo TripleQuadrupole - TQD (Waters MS Technologies, Manchester, U.K.) with an electrospray ionization ion (ESI) source was used for flavonoid detection. In positive ionization mode, the MS1 ranged from *m/z* 20 Da to 1974 Da. The parameters of the source for positive ionization mode were: cone voltage 40 V; source temperature 150 °C; electrospray capillary voltage 3.0 kV; desolvation temperature 300 °C; desolvation gas flow (L/h).

2.7. Statistical analysis

All samples were prepared in a quintuplicate. Data from UHPLC-HRMS were also explored using multivariate statistical analysis, MetaboAnalyst 5.0 (Xia Lab @McGill, Quebec, Canada) was used for principal component analysis (PCA).

3. Results and discussion

3.1. Annotation of flavonoids based on FBMN and correlation with pectin content

In this study, the primary aim was to investigate how pectin deficiency influences the availability of bioflavonoids. We utilized three distinct samples derived from pectin biorefinery, denoted as OPW_1, OPW_2, and OPW_3. Additionally, we included a reference sample, OPW_4, comprising untreated orange peels to serve as a basis for comparison. It is important to mention that all samples were produced in the same country, Brazil, all belong to the same variety of orange, and all were collected at the same maturity degree (since all were first used for juice extraction), which allows control of any variation that could come from these factors in flavonoids composition.

To estimate the pectin content in each sample, the *m*-hydroxydiphenyl method was employed. This method is highly sensitive and specific, once neutral sugars such as glucose, arabinose, fructose, rhamnose, galactose, and xylose present in cell walls do not cause interference (Ibarz et al. (2006)). The results were expressed as galacturonic acid equivalent (%, GalA equivalent) ± sd of three separate samples. GalA is the main uronic acid in the orange cell wall, which comes from pectic substances. The values found were: OPW_1 8.62 ± 0.20%, OPW_2 3.51 ± 0.98%, OPW_3 19.41 ± 0.05%, and OPW_4 20.42 ± 1.25%.

In the context of metabolites annotation, Feature-Based Molecular Networking (FBMN) emerges as an exceptionally potent approach for analyzing data derived from liquid chromatography high-resolution mass spectrometry (LC-HRMS/MS), particularly in the comprehensive evaluation of diverse flavonoid classes present in orange peel waste (OPW), the focus of this study. This methodology assumes a pivotal role in annotating, classifying, and comparing complex metabolites, with a specific emphasis on flavonoids. The evaluation of distinct classes of metabolites is enriched by the ability of FBMN to consider multiple parameters, including peak areas in mass spectra (Wang et al., 2020). The incorporation of this quantitative information into correlation networks allows not only the identification of flavonoids but also the evaluation of their relative concentrations in different samples. This quantitative approach further consolidates the usefulness of FBMN in comparative studies, enabling statistical analysis of variations in flavonoid expression between different experimental conditions or samples.

The comprehensive results of this study, including the data from the GNPS database, can be accessed through the following link: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=7c35a721113c4ca993a6608892a370b7. The selection of the best ionization method was done by counting the number of ions generated (features) after preprocessing the data in mZmine 3.9.0 (Schmid et al., 2023), with the positive ion mode being the choice for the annotation of the metabolites and assemblies of the FBMN (Fig. S5). The annotation of the different flavonoids was carried out using the spectral libraries used on the GNPS platform (Wang et al., 2016). For this study, the annotations were made only for the features that presented an MS2 profile, which was pre-defined according to the feature table generated by MZMine 3.9.0. The classification of flavonoids and other metabolites in this study followed the criteria established by Schymanski et al. (2014). These criteria encompass Annotation Level I, which confirms the structural identity through a direct comparison with reference standards; annotation Level II, involving the correlation of MS/MS fragments with spectra found within the GNPS database; annotation Level III, where structural evidence is based on *in silico* generated MS and MS/MS fragments; and annotation Level IV, reserved for annotated SMs where the molecular formula can be unequivocally determined using data from LC-HRMS analyses.

In this work, a total of 32 flavonoids from different classes were annotated using the positive ion mode. The flavonoids found in the samples belong to six classes: flavanone glycoside, 5; flavone glycoside, 6; flavanone aglycone, 3; isoflavone, 1; flavonol glycoside, 1; and, flavone polymethoxylated, 16. Notably, four of them achieved annotation Level I, with hesperidin, hesperetin, diosmin, and naringin being successfully confirmed using standards (as shown in Table 1). The other annotations presented in this study were carried out through different spectrometry databases and their comparative MS2 spectra can be accessed in the Supplementary Material (Figs. S12 - S32). Distinctive variations between the samples are visually observable in the chromatograms, as depicted in Fig. S10. The LC-HRMS/MS method employed was notably efficient, requiring 15-minute analysis, and proved suitable for annotation of a diverse range of flavonoids commonly found in oranges, encompassing both glycosides and aglycone compounds.

The identified flavonoids were thoughtfully organized into seven distinct maps, as depicted in Fig. S5. Within the context of the FBMN map, neighboring nodes represent structurally closely related compounds, revealing the well-established fragmentation patterns that characterize them. Hesperidin and sinesentin were taken as an example to show the fragmentation pattern in the positive ion mode of the flavonoid glycoside and aglycone, respectively. In the case of flavonoid glycosides, it is commonly observed the loss of the disaccharides structure. From hesperidin, the fragment ions at *m/z* 465 $[M-C_6H_{10}O_4+H]^+$ and at *m/z* 449 $[M-C_6H_{10}O_5+H]^+$, by the loss of 146 and 162 Da are observed, indicating the loss of rhamnose and glucose, respectively. Also, the fragment at *m/z* 303 is observed, related to hesperetin, the basic nucleus of this flavanone (Xia et al., 2023; Cuyckens & Claeys, 2004). In the case of sinesentin, a polymethoxylated flavonoid, the common fragment generated at low collision energy is O-methyl. Also,

Table 1
Flavonoids annotated from the samples by positive mode ionization analysis combined with FBMN processing.

N°	*m/z* theoretical	*m/z* experimental	Error (ppm)	Rt (min)	Formula	Annotations	Class	Distribution
1	625.1763	625.1763	0	4.27	$C_{28}H_{32}O_{16}$	Diosmetin 3,8-di-C-glucoside	Flavone	All
2	465.1390	465.1390	–	4.54	$C_{18}H_{18}O_7$	Hesperetin 7-O-glucoside	Flavanone	All
3	565.1551	565.1551	–	4.58	$C_{26}H_{28}O_{14}$	Isovitexin 2″O-arabinoside	Flavone	All
4	579.1708	579.1709	0.3453	4.64	$C_{27}H_{30}O_{14}$	Rhoifolin	Flavone	All
5	597.1813	597.1816	0.3349	4.67	$C_{27}H_{32}O_{15}$	Eriocitrin	Flavanone	All
6	463.1234	463.1234	0	4.79	$C_{22}H_{22}O_{11}$	Dalpanitin	Isoflavona	All
7	579.1708	579.1709	−0.1727	4.88	$C_{27}H_{30}O_{14}$	Compound NP-003829	Flavone	All
8	581.1864	581.1865	0.1720	4.90	$C_{27}H_{32}O_{14}$	Naringin* or Naringenin-7-rutinoside	Flavanone	All
9	609.1815	609.1814	0.1641	4.98	$C_{28}H_{32}O_{15}$	Diosmin*	Flavone	All
10	611.1970	611.1969	−0.1636	5.05	$C_{28}H_{34}O_{15}$	Hesperidin*	Flavanone	All
11	449.1442	449.1442	0	5.09	$C_{22}H_{24}O_{10}$	Sakuranetin-5-*O*-glucoside	Flavanone	All
12	595.1657	595.1657	–	5.59	$C_{27}H_{30}O_{15}$	Kaempferol-3-O-rutinoside or Vicenin II	Flavonol	OPW_2, OPW_4
13	595.2021	595.2020	0.1680	5.65	$C_{28}H_{34}O_{14}$	Poncirin or Neoponcirin (Dyimin)	Flavanone	All
14	359.1125	359.1124	−0.2784	6.02	$C_{19}H_{18}O_7$	3-Hydroxy-4′,5,6,7-tetramethoxyflavone	PMF	All
15	273.0757	273.0755	−0.7323	6.06	$C_{15}H_{12}O_5$	Naringenin	Flavanone aglycone	All
16	329.1016	329.1018	0.6077	6.12	$C_{18}H_{16}O_6$	Monohydroxytrimethoxyflavone	PMF	OPW_1, OPW_4
17	303.0863	303.0860	−0.9898	6.23	$C_{16}H_{14}O_6$	Hesperetin*	Flavanone aglycone	All
18	315.0863	315.0862	−0.3173	6.70	$C_{17}H_{14}O_6$	Cirsimaritin	PMF	All
19	373.1282	373.1282	0	6.73	$C_{20}H_{20}O_7$	Isosinensetin	PMF	All
20	403.1387	403.1387	–	7.02	$C_{21}H_{22}O_8$	Nobiletin	PMF	All
21	343.1176	343.1175	−0.2914	7.13	$C_{19}H_{18}O_6$	Tetramethylscutellarein	PMF	All
22	287.0914	287.0913	−0.3483	7.14	$C_{16}H_{13}O_5$	Ponciretin	Flavanone aglycone	All
23	375.1074	375.1076	0.5331	7.16	$C_{19}H_{18}O_8$	5,2'-dihydroxy-6,7,8,6'-tetramethoxyflavone	PMF	All
24	433.1493	433.1493	0	7.33	$C_{22}H_{24}O_9$	3,5,6,7,8,3′,4′-Heptamethoxyflavone	PMF	All
25	359.1125	359.1124	−0.2784	7.40	$C_{19}H_{18}O_7$	Gardenin B	PMF	All
26	419.1337	419.1335	−0.4771	7.46	$C_{21}H_{22}O_9$	2-(3,4-Dimethoxyphenyl)-5-hydroxy-3,6,7,8-tetramethoxychromen-4-one	PMF	All
27	373.1282	373.1282	0	7.50	$C_{20}H_{20}O_7$	Sinensetin	PMF	All
28	359.1125	359.1123	−0.2784	7.59	$C_{19}H_{18}O_7$	3-Hydroxy-4′,5,6,7-tetramethoxyflavone	PMF	All
29	389.1230	389.1229	−0.2569	7.76	$C_{20}H_{20}O_8$	5-O-Demethylnobiletin	PMF	All
30	329.1018	329.1018	−0.3038	7.86	$C_{18}H_{16}O_6$	Monohydroxytrimethoxyflavone	PMF	OPW_1, OPW_4
31	419.1337	419.1336	−0.4771	8.01	$C_{21}H_{22}O_9$	2-(3,4-Dimethoxyphenyl)-5-hydroxy-3,6,7,8-tetramethoxychromen-4-one	PMF	All
32	359.1125	359.1124	−0.2784	8.20	$C_{19}H_{18}O_7$	3-Hydroxy-4′,5,6,7-tetramethoxyflavone	PMF	All

Notes: [a]All fragmentation information is contained in the Supplementary Material; *This superscript represents standard references.

the losses of -CH_3 and H_2O are other possible pathways of sinesentin fragmentation (Wen et al., 2021; Zhang et al., 2013).

In OPW_1, the flavonoids - hesperidin (*m/z* 611.197), naringin (*m/z* 581.1864), hesperetin 7-O-glucoside (*m/z* 465.1395), and hesperetin (*m/z* 303.086) are present in higher concentrations than in OPW_2. The flavonoids classified as flavanones, were grouped in the same cluster (Fig. S6). The MS/MS data of hesperidin $[M + H]^+$ display the ion related to its basic skeleton (hesperetin) at *m/z* 303.09 $[M-\text{rutinoside} + H]^+$ and others (Fig. S3). For naringin, hesperetin 7-O-glucoside, and hesperetin, the MS/MS spectra show the *m/z* at 273.08 from $[M-\text{glucoside} + H]^+$, *m/z* 287 $[M-CH_3]^+$, and *m/z* 303 $[M-\text{glucose} + H]^+$, respectively (Cuyckens & Claeys, 2004).

On the other hand, the flavones glycosides diosmin (*m/z* 609.1814), kaempferol-3-O-rutinoside (Vicenin II) (*m/z* 595.1657), and NP-003829 (*m/z* 579.1707), are found at significantly elevated levels in OPW_2 compared to the other OPWs (as shown in Fig. 1, A). In the case of the diosmin, the fragments at *m/z* 463.12 and 301.07 refer to the losses of $[M-C_6H_{10}O_4 + H]^+$, and $[M-\text{rhamoglucosyl} + H]^+$, respectively (Fig. S1). For Vicenin II, the main fragments were at *m/z* 449.1077 and 287.0551, which represent the loss of rhamnose and rhamnoglucoside, respectively (Fig. S12). Finally, for NP-003828, the fragments observed were those at *m/z* 433.1137 $[M-C_6H_{10}O_4 + H]^+$, and *m/z* 271.0603 $[M-C_{12}H_{20}O_9 + H]^+$ (Fig. S13). To show the distribution of these compounds among the samples, the peak area values were calculated for all OPWs and then plotted on a bar chart. The results are an average of the chromatogram areas of five independent biological replicates, expressed as mean ± standard deviation. A statistical analysis was carried out using a *t*-test to compare the different OPW samples (see Fig. 1, B). In addition to these flavonoids, eriocitrin (*m/z* 597.1813) shows a substantial concentration in OPW_2 (Fig. S6).

The polymethoxylated flavonoids (PMFs), specifically, flavones, were found mostly in the OPW_3 and OPW_4 and, in a minor extension in OPW_1 (Fig. 2). 16 polymethoxylated flavones were found in the samples. In this molecular family of flavonoids, there are 24 nodes. The polymethoxylated flavones were 5,2'-dihydroxy-6,7,8,6'-tetramethoxyflavone (*m/z* 375.1074), 3,5,6,7,8,3′,4′-heptamethoxyflavone (*m/z* 433.1493), nobiletin (*m/z* 403.1387), 3-hydroxy-4′,5,6,7-tetramethoxyflavone (*m/z* 359.1125), isosinensetin and sinensentin, 2-(3,4-dimethoxyphenyl)-5-hydroxy-3,6,7,8-tetramethoxychromen-4-one or gardenin A (*m/z* 373.1282), 5-O-demethylnobiletin (and its isomer), (*m/z* 389.1230), tetramethylscutellarin (*m/z* 343.1176), monohydroxytrimethoxyflavone (*m/z* 329.1016) (Figs. S21–S29). These findings were following Xing, Zhao, Zhang, and Li (2017). These authors investigated the PMFs in citrus peels from *C. reticulata* and *C. sinensis,* and found that most *C. sinensis* samples contained fewer than 20 PMFs. In this investigation, the distinctive fragmentation pattern of PMFs can be observed through the fragmentations that occur in this category of molecules, including the loss of $-CH_3$, H_2O and $-OCH_3$. These losses were crucial for grouping the characteristic nodes of the annotated molecules (Wen et al., 2021) belonging to the PMF classes, as shown in Fig. 2.

The relative distribution of the flavonoid subclasses in each sample based on the average of the chromatogram area is presented in Fig. 3. As can be observed, flavonoid glycosides were most prominently concentrated in the by-product resulting from the pectin extraction process, mainly in OPW_2. Although OPW_1 is a sample from the pectin industry, its profile is very similar to that of the samples without treatment (OPW_4). The percentage of flavanone aglycone in this sample (8%) is the

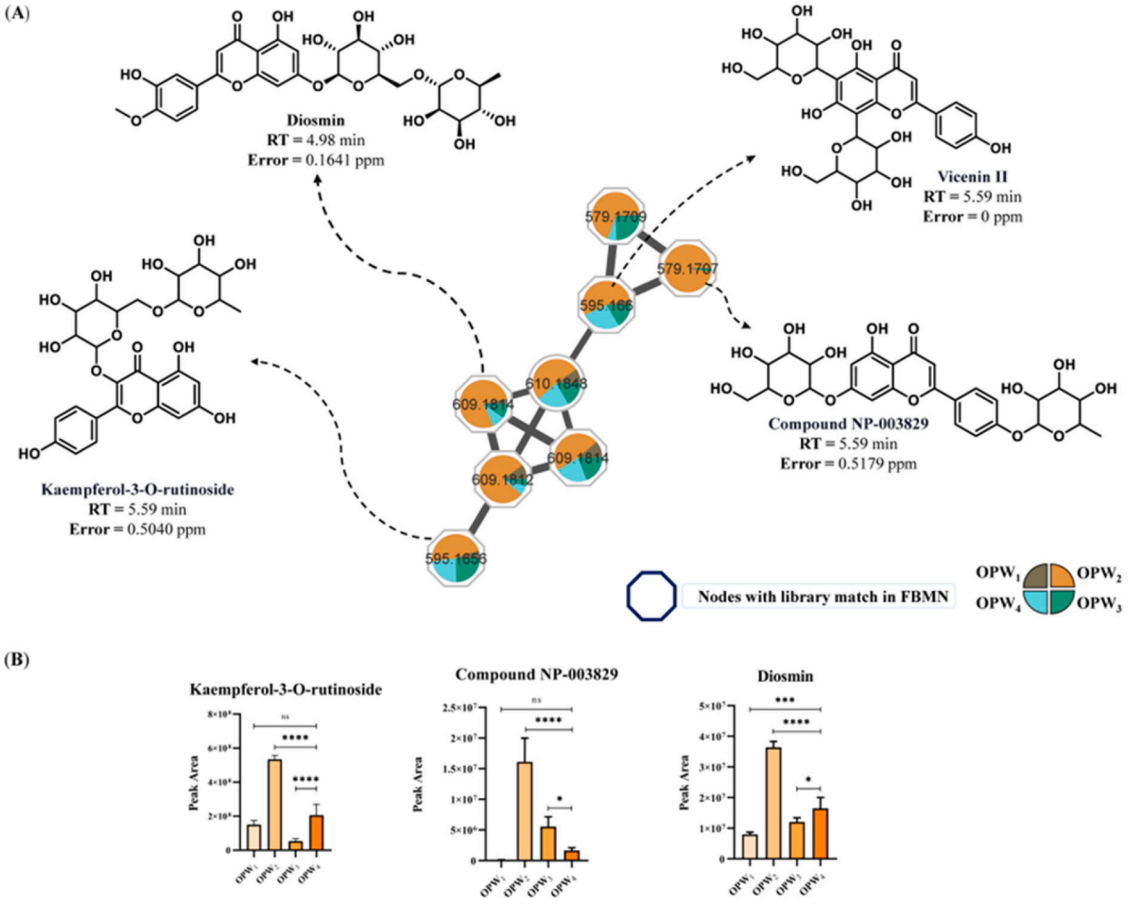

Fig. 1. (A) Molecular network map of some flavonoid glycosides in OPWs. (B) Relative abundances of some flavonoid glycosides based on peak area. The statistical significance level is denoted as follows: ns – *p-value* > 0.05; * - *p-value* < 0.05; ** - *p-value* < 0.01; *** - *p-value* < 0.001; **** - *p-value* < 0.0001.

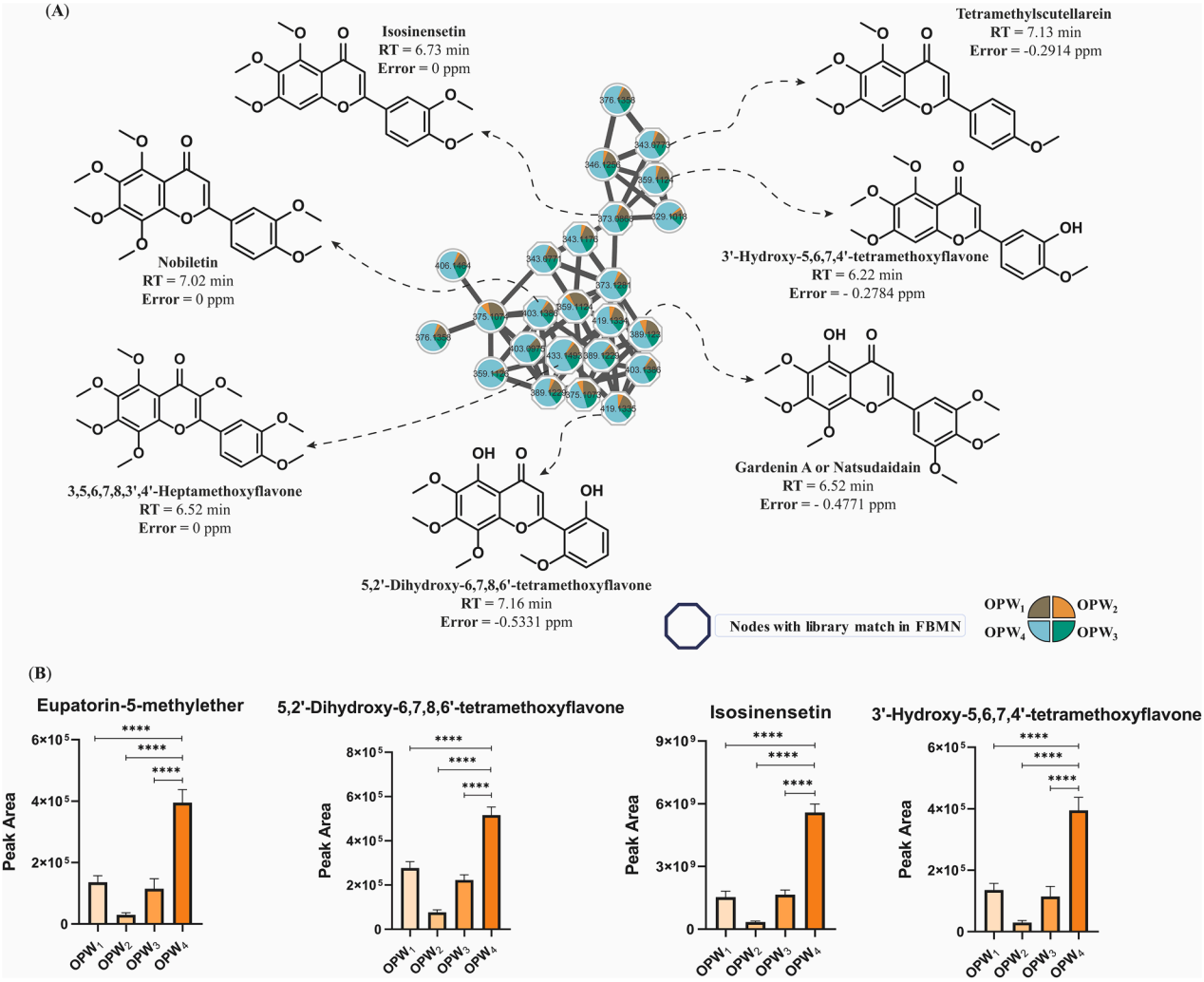

Fig. 2. (A) Molecular network map of some polymethoxylated flavonoids in OPWs. (B) Relative abundance of some polymethoxylated flavonoids based on peak area. The statistical significance level is denoted as follows: ns – *p-value* > 0.05; * - *p-value* < 0.05; ** - *p-value* < 0.01; *** - *p-value* < 0.001; **** - *p-value* < 0.0001.

main difference between this sample relatively through the others. Flavones polymethoxylated in the aglycone form were more common in OPW_3 and OPW_4, 89% and 82%, respectively. The comparisons between the samples allow us to observe that OPW without acid treatment is a rich source of flavones polymethoxylated and OPW from a pectin factory is enriched with flavonoid glycosides. Besides, it is possible to verify that the internal tissue of the oranges (OPW_3) is the principal source of polymethoxylated flavones.

These results highlight the possibility of different interactions between flavonoids and pectin and/or different stability between flavonoids throughout the classes. It is worth mentioning that when food materials are processed, the flavonoids, located in the vacuole of the plant cells, enter into contact with the plant cell walls starting a variety of different interactions (Renard, Watrelot, & Bourvellec, 2017). However, the chemical characteristics of phenolic compounds imply different kinds of interactions within the plant cell walls (Phan et al., 2015). These interactions may occur through hydrophobic interactions, electrostatic interactions, hydrogen bonds, and as shown recently, via bound iron molecules. The presence of C2-C3 bond in flavonols was responsible for improving the binding of this class of flavonoids with pectin in the presence of iron ions, as suggested by Chirug, Nagar, Okun, and Shpigelman (2021). These authors investigated the interactions between quercetin and rutin (the glycoside of quercetin) with iron-enriched water-soluble pectin and verified that the sugar-binding to rutin might reduce the number of possible site interactions, which explains its low affinity with pectin. These findings suggest that the presence of metal ions influences phenolics and plant cell wall interactions. Based on the results achieved by this study (low concentration of flavonol aglycone in pectin by-product - OPW_2), and the findings of these authors, an adequate explanation is that flavonoids aglycones bind to pectin and then are extracted together with this polysaccharide.

Studies show that hesperidin, a well-known flavanone, is insoluble and stable in acid conditions (Majumdar & Srirangam, 2008). Hesperidin belongs to the flavanone class and it is a flavanone glycoside. Another study reported by Biesaga (2011) shows the influence of four types of solvent and extraction methods on flavonoid stability and found that sugars attached to the flavonoids protect them from degradation when submitted to microwave and ultrasound extraction. In this sense, these characteristics could be extended to all flavonoids in the glycoside form, considering the similarity between them. This fact could be an explanation for the highest amount of these kinds of flavonoids in the pectin by-product samples. The implication is that flavonoid glycosides retained their structure even after strong conditions of the acid pectin extraction. On the other hand, flavonoid aglycones seem to be much more sensitive to acid and high-temperature conditions. However, there is a lack in the literature related to flavonoid aglycones' stability to acid and temperature conditions to support this hypothesis. We hypothesized that these factors together may have implied the differences observed between OPW flavonoid profiles. The relatively lower amount of flavonoids aglycone in the samples from the pectin industry (OPW_1 and

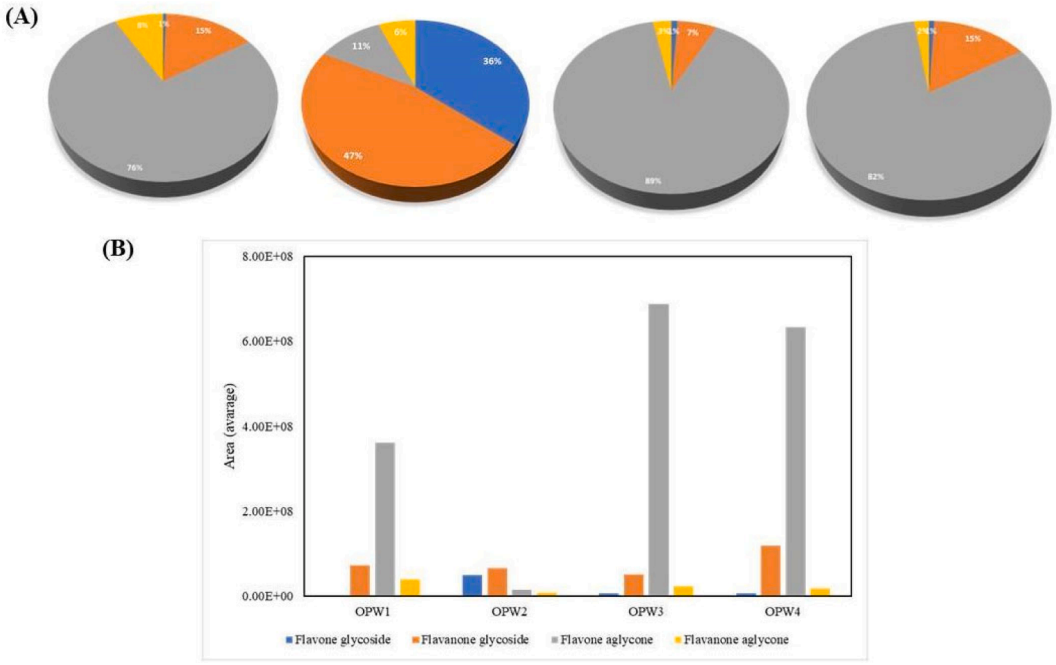

Fig. 3. (A) Relative distribution of flavonoid subclasses in the OPWs samples. From left to right, OPW_1, OPW_2, OPW_3 and OPW_4. The color refers to flavone aglycone, grey; flavanone aglycone yellow; flavone glycoside blue; and flavanone glycoside orange. (B) Bar graph showing the relative flavonoid distribution through the samples.

OPW_2) proves the lower stability of these flavonoids through acid and high-temperature conditions, and also the preferential interaction of these with pectin.

Besides, these results show that pectin by-products could be a source of attention of flavonoid glycosides, like hesperidin, diosmin, eriocitrin, vicenin II, and kaempferol-3-O-rutinoside. These flavonoids are known for their benefits for human health. Diosmin, for example, is known for its potential therapeutic benefits and is often used as a dietary supplement or medication for various health purposes. It is commonly used in the treatment of venous disorders, particularly chronic venous insufficiency and hemorrhoids. Diosmin helps improving the tone and elasticity of blood vessels, reducing venous stasis and related symptoms like leg swelling and pain. Also, diosmin shows antidiabetic properties (Mustafa et al., 2022; Thanapongsathorn & Vajrabukka, 1992).

3.2. Flavonoids quantification

Four selected flavonoids were quantified in OPW samples by MS response using the regression equations shown in Table 2. The retention time (Rt, min) for each compound was 4.88 for hesperidin, 4.75 for naringin, 4.81 for diosmin and 5.85 for hesperetin. Although the positive and negative modes were accomplished in the analysis, the positive ion mode was selected for the quantification. All correlation coefficients were higher than 0.99. The results from the accuracy of the method were: 95.07 ± 12.44% for hesperidin, 107.22 ± 22.31% for hesperetin, 100.68 ± 7.32% for diosmin and 99.29 ± 7.04% for naringin. The results were expressed as g/100 g on a dry basis for hesperidin and naringin, and as mg/100 g on a dry basis for diosmin (Table 3). For hesperetin, the values were under the limit of quantification - LOQ to OPW_2, OPW_3 and OPW_4.

To the best of our knowledge, it is the first report about the quantification of these compounds by MS response in OPW samples. The usual method for flavonoid quantification is HPLC coupled with a UV detector (DAD). Xia et al., 2023, for example, quantified some flavonoids in *Citrus medica* using UHPLC-DAD. Omoba, Obafaye, Salawu, Boligon, & Athayde, 2015 another example of the use of HPLC-DAD for flavonoid quantification in citrus. The MS method has many advantages through UV detectors, like higher sensitivity, specificity, and accuracy. Also, MS detectors typically have a wider dynamic range than DAD detectors, allowing for the quantification of both low and high concentrations of flavonoids within the same analysis (Xing, Zhao, Zhang, & Li, 2017). The quantification results showed, for example, that diosmin is present in higher concentrations in OPW_2,whereas hesperidin and naringin are

Table 2
Linearity of standard curves for reference flavonoids.

Compounds	Regression equation	R^2	Linear range (μg/mL)	LOQ* (μg/mL)	LOD** (μg/mL)
Hesperidin	$y = 13547x - 209.38$	0.996	0.250–10.0	1.66	0.52
Diosmin	$y = 61181x + 281.65$	0.999	0.015–1.0	0.14	0.02
Hesperetin	$y = 3509.9x - 283.82$	0.996	0.015–1.0	0.35	0.22
Naringin	$y = 11447x + 1650.9$	0.997	0.250–10.0	1.30	0.39

*LOQ - limit of quantification; **LOD - limit of detection.

Table 3
Content of common flavonoids in analyzed OPW.

Compounds	Orange Processing Waste - OPW			
	OPW_1	OPW_2	OPW_3	OPW_4
Hesperidin*	$2.14^{a} \pm 0.34$	$1.74^{b} \pm 0.09$	$1.60^{bc} \pm 0.25$	$2.40^{d} \pm 0.43$
Naringin*	$1.16^{a} \pm 0.15$	$0.72^{b} \pm 0.09$	$0.90^{ab} \pm 0.17$	$2.04^{c} \pm 0.25$
Diosmin**	$11.10^{a} \pm 0.59$	$62.95^{b} \pm 5.29$	$33.34^{c} \pm 5.23$	$15.27^{ad} \pm 2.26$
Hesperetin**	9.31 ± 0.82	–	–	–

*Values expressed as g/100 g; **Values expressed as mg/100 g; Statistical note: Means (n = 5), different letters in the same line means significant difference, 95% ($p < 0.05$) according to Tukey's range test, and superscripts with the same letter are not significantly different.

more abundant in OPW_1 and OPW_4.

3.3. Multivariate analysis

The multivariate analysis of the LC-HRMS dataset was performed to highlight the potential discriminatory metabolites between the samples. The technique used was Principal Component Analysis (PCA), an unsupervised pattern recognition method. PCA is a statistical technique used for reducing the dimensionality of data while preserving the most important information. It accomplishes this by transforming the data into a new coordinate system where the axes are aligned with the directions of maximum variance in the original data (Cumpson et al., 2015). Two PCA score plot analyses were performed, one including the blanks and the QCs (Fig. S11, A) and another without blanks and QCs (Fig. S11, B). When blanks and QCs were included in the PCA analysis, the two PCs explained 51.4% of the cumulative variance whereas when it did not include the two PCs explained 59.7%. In both cases, OPW_1 and OPW_2 samples were clustered at negative values of PC1 and positive values of PC2. In the case of OPW_3 samples were clustered at positive values of PC1 and negative values of PC2. OPW_4 samples were clustered at positive values of PC1 and PC2. Loadings graph of the samples without blanks and QCs revealed that flavonoid glycosides contribute to distinguished samples from the pectin industry, specifically diosmin, compound NP-003829, kaempferol-3-O-rutinoside and eriocitrin from OPW_2. On the other hand, flavonoids polymethoxylated were responsible for the clustering of the OPW_3 and OPW_4 samples. Besides flavonoids, other compounds were responsible for the separation of the samples. For example, the coumarins 5,8-dimethoxypsoralen, 8-geranyloxypsoralen, oxypeucedanin hydrate, and isoimperatorin are at high levels in OPW_3. Also, in OPW_2, bergaptol is present at a high level (Fig. S7 and S8).

3.4. Biorefinery proposal

Since OPWs from pectin factories are a rich source of flavonoid glycosides, a possible approach for better utilization of these waste would be the extraction of these compounds (Fig. 4). If the yield of pectin extracted industrially reaches 10%, then from 100 g of orange peels remaining at the end about 90 g of waste, which means that the amount of waste generated at the end of the pectin extraction is high. In this sense, it is a sustainable source of flavonoids for extraction. Taking hesperidin as an example, 100 g of the OPW from the pectin industry can render about 2 g of this compound. The price of 25 g hesperidin with purity > 80% is approximately US$ 116. The hesperidin market forecast value in 2031 is estimated at US$ 384.3 million, with a growth rate of 7.3% from 2021 to 2031 (Hesperidin Market, 2023). The global flavonoid market is experiencing growth due to increased consumer awareness of the health benefits associated with flavonoid-rich diets. Factors such as the rising demand for natural and plant-based ingredients, the growth of the dietary supplements market, and the trend towards healthier eating contributed to the market's expansion (Global Market, 2023).

Flavonoids found applications in various sectors, including dietary supplements, pharmaceuticals, cosmetics, and the food and beverage industry. They are often included in products like teas, nutritional supplements, and skincare items due to their potential antioxidant and anti-inflammatory properties. In addition to the interest in the therapeutic potential of these flavonoids, their abundance in industrial waste, such as OPW_2, could also have significant implications for agriculture. Flavonoids such as diosmin and eriocitrin are known for their antioxidant properties and their role in protecting plants against environmental stresses such as UV radiation and pathogens. These compounds can serve as natural agents to strengthen plant resistance, reduce the need for chemicals and improve crop quality. In addition, studies have shown that flavonoids can play a fundamental role in promoting plant growth, stimulating nutrient absorption and optimizing metabolic processes (Lidoy et al. (2023)). Flavonoids show intriguing potential for the development of more sustainable phytosanitary products, as certain varieties have demonstrated pesticidal activity. Therefore, the high content of these flavonoids in all OPWs could open up new perspectives in agriculture, contributing to more sustainable and effective farming practices.

In the context of the sports supplements industry, the incorporation of flavonoids emerges as a promising strategy to support not only muscle recovery but also the overall health of athletes. Furthermore, their versatility extends to the manufacturing of personal care products such as creams and lotions, owing to their beneficial properties for the skin. This adaptability exemplifies the broad spectrum of applications for flavonoids in multiple sectors, reflecting the growing demand for natural and healthy solutions across various industries (Chauhan, Pandit, Mohanty, & Meena, 2023).

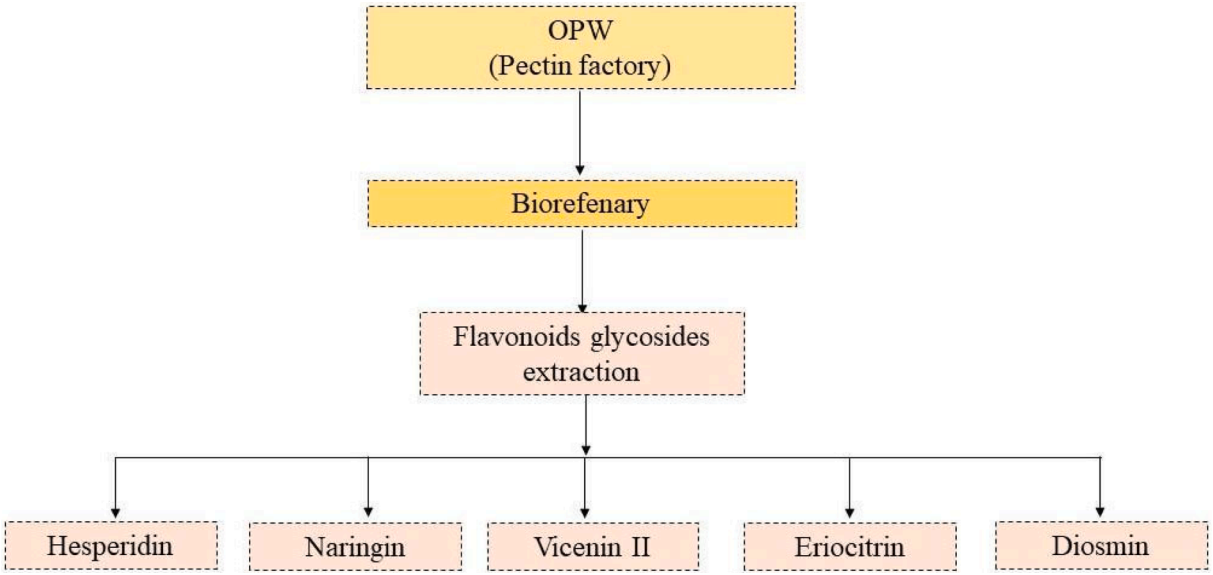

Fig. 4. Proposed biorefinery scheme for OPW from Pectin factory.

4. Conclusions

In conclusion, our study harnessed the power of LC-HRMS/MS and FBMN to examine the flavonoid profiles within samples originating from pectin factories (OPW_1, OPW_2, and OPW_3), contrasting them with untreated orange peels (OPW_4). The FBMN approach allowed the annotation of a total of 32 flavonoids across Levels I and II, comprising 17 polymethoxylated flavonoids, 11 glycoside flavonoids, and 2 aglycone flavanones. Remarkably, our results underscore the prevalence of flavonoid glycosides within the samples from the pectin factories, even in the face of rigorous extraction conditions involving acids and elevated temperatures. Traditionally, pectin extraction from citrus peels follows the essential oil extraction process, with the residual material often relegated to waste or repurposed as animal feed. However, our findings suggest a more sustainable and valuable alternative. By optimizing the extraction of glycoside flavonoids from this resource, we can unlock its untapped potential. For instance, from every 100 g of OPW_1, we can extract a substantial 2.1 g of hesperidin and 62.95 mg of diosmin. Notably, these flavonoids hold high intrinsic value in the market, providing this approach not only ecologically soundness but economical viability. Our research paves the way for the efficient utilization of this overlooked resource, promoting both environmental sustainability and economic prosperity.

CRediT authorship contribution statement

Symone Costa de Castro: Writing – original draft, Investigation, Formal analysis. **Júlio César Jeronimo Barbosa:** Writing – original draft, Methodology, Investigation, Formal analysis. **Bruno Sozza Teixeira:** Writing – original draft, Formal analysis, Data curation. **Taicia Pacheco Fill:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Ljubica Tasic:** Writing – review & editing, Resources, Investigation, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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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.2024.101326.

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