NEW TECHNOLOGY FOR OBTAINING FLAVONOIDS FROM MANDARIN PROCESSING WASTE

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Abstract

The mandarin (C. unshiu) is widely distributed in Georgia. Natural juices and concentrates are mainly made from this fruit. During processing, more than 60% of mandarin pomace (MP) is obtained as production waste and released into the environment, causing harm to nature. However, waste is also a source of functional ingredients, including important flavonoids that impact human health. In this study, we examined the technological parameters of mandarin flavonoid pomace (PEF). An extractor model was created and the influence of the mixing intensity of a mandarin pomace and extractant mixture (water and calcium oxide) on the flavonoid extraction rate and yield was determined. Optimal extraction parameters were found to be a speed of 500 rpm for 2 hours, increasing the yield by 12%. The quantitative content of flavonoids was determined using a mixture of the extractant dimethyl sulfoxide (DMSO) and ethanol (10:2) by the spectral method (286 nm). To assess the flavonoids qualitatively, a purification method was developed using a mixture of 10% NaOH and 96% C_2H_5OH in a 1:1 ratio, followed by further processing in the CO_2 zone. The quantitative content of hesperidin in the resulting preparation is greater than 90%. Based on the research results, a rational technological scheme has been developed for enterprises producing flavonoid extracts.

Keywords: industrial mandarin waste, UPLC-MS analysis, flavanoid extract pomace (FEP), Hesperidin

I.Introduction

It is known that citrus fruits (Rutaceae), which include oranges, tangerines, limes, lemons, sour oranges (Bigaradia), and grapefruits, contain many substances useful for the human body [1]. To date, citrus fruits are distributed in 140 countries of the world, namely in both belts of the earth's equator, covering the tropical and subtropical regions of the world between 35 ° south latitude and 35 ° north latitude. Most of the harvest and production is concentrated in the Southern Hemisphere [2]. World cultivation of citrus fruits has grown significantly in recent years, and their annual production has reached 110 million tons [3]. In Georgia, which is practically the extreme north of citrus distribution, mandarin (C. Unshiu) is predominant. Its average annual yield is 100 thousand tons.

Citrus fruits are characterized by flavonoid glycosides: hesperidin, naringin, neoheperidin. Although more than 5000 flavonoids are known [10], only a limited number of them have been

determined by their effect on the appearance, taste and nutritional value of the product. For example, an excess of hespedin in citrus juice causes puffiness [11], and naringin causes a feeling of bitterness [12].

In citrus juices, we have identified and quantified flavonoid compounds, in the core of which the hydroxyl is located mainly in positions C-5 and C-7 [13].

Flavanones are characterized by a wide range of biological and pharmacological activity (antioxidant, anticarcinogenic, anti-inflammatory), they play an important role in the prevention of bone loss, cancer and atherosclerosis [6,7].

The high content of biologically valuable compounds in citrus residues has led to great interest in waste valorization. Researchers use citrus waste in a variety of beneficial ways to minimize environmental damage. Citrus residues are rich in phytochemicals [8]. Therefore, it is advisable to use citrus residues for functional nutrition and food preparation [9].

To date, the most common and effective method for the study of phenolic compounds is the HPLC method. In most cases, their detection and quantitative study is possible without prior derivatization and concentration. Reverse phase chromatography is widely used to separate flavonoids on C8 or C18 columns (using a gradient of polar solvent mixtures). Gradient chromatography is also often used to separate flavonoids [14,15,16,17].

The main product of industrial processing of mandarin fruits in Georgia is juice and its concentrate. Juice production wastes (peel, part of the pulp, seeds), despite the fact that they contain a wide range of secondary components such as polyphenols, carotenoids, vitamins, dietary fiber, etc., unfortunately, are thrown away and remain unused [2,3, 4, 5]. It should be noted that the biologically active substances of mandarin grown in Georgia and the possibility of their use using modern research methods have not been fully studied.

II. Materials and Methods

2.1. Reagents and Samples

Hesperedin (standard), Chemical reagents Acetonitrile, Formic acid, were purchased from Sigma–Aldrich (Germany; Tbilisi, Georgia)

Mandarin extract - produced from raw mandarin fruits grown in Western Georgia, Unshiu variety (Citrus unshiu L), harvest 2020-2021. The industrial technological process of juice preparation provides for the selection and checking of fruits, washing, obtaining juice on a roller press (the fruit is cut in half, held on a perforated surface), then the residue is additionally squeezed out on a screw press. The juice preparation process was carried out on the technological line of the Italian production "FENCO". The production process was carried out at a citrus processing plant (Sakrtvelo, Kobuleti), owned by Georgian Industrial Asset Management Group LLC. For research, there has been used an extract, obtained from the press.

Preparation of FEP samples.

Pre-washed mandarin juice, crushed to particles 0.5-1.0 mm in size, was placed in an extractor equipped with a weeder (rotation speed 50-1000 rpm) and a temperature controller (0-1000C), drinking water was added (1 part of the juice and 2 parts of water), calcium oxide was added until the pH of the mixture reached 11. The test mixture thus prepared was divided into four samples of equal weight. Exposure of sample No. 1 (control) for 24 hours with periodic stirring (4-5 times); A mixture of the remaining samples (No. 2, No. 3, No. 4) was loaded into the extractor, intensively mixed at a speed of 355, 500 and 710 rpm, respectively; Samples were processed at a temperature of +350C. In addition to the control, the test samples were processed for different durations at hourly intervals from 1 to 5 hours. For all samples after the extraction process, the suspension was removed, filtered and 20% hydrochloric acid was added until the pH value of the region was 5. The acidified extract was kept at a temperature of +6-(+8) ° C for 24 hours until the flavonoid complex was completely precipitated. At the end of the process, the supernatant was drained with a siphon, and

the remaining mass was centrifuged at 3000 rpm at a temperature of +8^{0}C. There has been formed a pale precipitate, which was dried at 60-65^{0}C and then crushed; dry PFE has been obtained.

2.2.Methods

Standard methods of analysis

Determination of dry matter - by thermogravimetric method (AOAC Official Method); **Quantitative determination of water-soluble dry substances** - by means of a digital refractometer (Carl Zeiss, Germany) (AOAC Official Method, **920.151 (37.1.12)(AOAC, 2000).**

Determination of total titration acidity and pH value - on an automatic titrometer (Mettler-Toledo AG, Analytical www.mt.com/education-line CH-8603 Schwerzenbach, Switzerland) **920.151 (37.1.12)(AOAC, 2000)**;

determination of carotenoids - by HPLC method ~~(19)~~;

determination of total pectin by carbazole - spectral method (26);

Quantitative determination of vitamin C - using the iodometric method of titration (AOAC Official Method, 1999, Gaithersburg, MD, method 939.13 and 966.18)and HPLC (25);

Fiber - by AOAC method (AOAC, 2000);

Quantification of carbohydrates – by HPLC Method;

2.2.1. Hesperidin separation and identification of substances by Ultra High-Performance Liquid Chromatography (UPLC)-PDA,MS methods; **(**Waters, Ultra-high pressure liquid chromatography system, Waters PDA Deteqtor, Acquity UPLC H-Class Core System, Acquity QDa Single Quadupole Mass-Detector, USA) analysis by column BEN HSS, 1.7 μm, Eluent Water+0.1 % F.A. (A) and ACN+0.1% F.A. (B), (gradient 0 min-2% B; 0-3 min 25% B, 3-6 min 26% B, 6-10 min 35% B, 10-12.5min 99% B, 12.5-13min 99% B, 13-13.5 min 2% B, 13.5-15 min 2 % B), Flow 0.3 ml/min, column tem. 40°C, MS scan 100-1200 da, Probe 600°C, Positive (ESI-MS)+ ცხრ negative (ESI-MS)-, capillary 0.8 kV, CV -15.

Qualitative and quantitative study of vitamin C by high pressure liquid chromatography method. Instrument - Waters Binary HPLC Pump 1525; Breeze 2489, detector - ultraviolet and visible lights, column - C18 Bridge (150 mm x 4.8 mm), eluent - water: methanol (50:50), detection at 270 nm (25).

Determination of carbohydrate by High performance liquid chromatography (HPLC RI) - Waters (Waters HPLC Pump 1525), chromatographic column with amide (250 mm x 4.5 mm); column temperature 40^{o}C, eluent 80% acetonitrile detected by refractometry (Waters RI detector 2414).

Quantification of hesperidin

For the quantitative determination of hespedin, we have used a spectrophotometric method. The quantitative study is based on the determination of the optical density of flavanones, hesperidin has been used as a standard sample. Flavonoids have been extracted with a mixture of ethyl alcohol and dimethyl sulfoxide (DMSO) (10:2). Using this extractant, hesperidin was extracted from the samples and spectrophotometry was performed at 286 nm [20].

An analytical sample of the raw material was crushed to a particle size passing through a 2 mm sieve. A sample weighing about 1.0 g (exact weight) was placed in a 100 ml flask.

To build a calibration curve, we transferred 0.01 g of standard hesperidin into a 50 ml volumetric flask, added 40 ml of a mixture of solvents, dissolved hesperidin by shaking, and filled the flask to the mark.

The following volumes of the standard solution were transferred into flasks with a capacity of 25 ml: 0.6; 1.0; 1.4; 1.8; 2.2; 2.6; 3.0; 3.4; 3.8 ml, the flasks were filled to the mark with a mixture of solvents, the concentration of hesperidin were: 0.48; 0.80, 1.12; 1296; 1.76; 2.08; 2.40; 2.72; 3.04*10-5 g/ml. An analytical solution was poured into the working cuvette, and a mixture of solvents was

poured into the comparative cuvette. The calibration graph of standard solutions, built depending on the optical density of the solutions (D) and the quantitative indicators of various concentrations of hesperidin (C), had the form of a straight line.

The mass fraction of flavonoids in the studied extracts in terms of hesperidin has been calculated by the following formula:

$$X= \frac{c*100*25*100}{a*1*(100-w)}*100\%;$$

where:
c - concentration of the analyzed solution, corresponding to the measured optical density on the calibration curve - 10^{-5} g/ml.
a - mass of raw materials, g;
w - loss of raw materials during drying, %.

Qualitative assessment of FEP

The extraction of hesperidin from the flavonoid extract has been carried out according to the following method: 5 g of the dry preparation was dissolved in an equal mixture of 10% sodium alkali solution and 96% ethyl alcohol. Dissolution occured within 15 minutes with constant shaking. It was then filtered under vacuum. The resulting filtrate was subjected to a flow of carbon dioxide for 1.5 hours, whereby hesperidin was abundantly precipitated. It was then filtered under vacuum; the precipitate remaining on the filter was first treated with a weak solution of hydrochloric acid and then washed with hot water. The resulting hesperidin was a colorless microscopic needle.

Statistical Analysis – Standard error was calculated for each data using Excel software. Confidence coefficient $p \leq 0.05$.

III. Results and discussion

Mandarin, like other citrus fruits, is characterized by a high biological value due to its special chemical composition (21,22). Hesperidin has been found mainly in the peel of citrus fruits and is considered to be the main functional component. Hesperidin dominates among the flavonones in the peel of mandarin fruits (23), whit has also been confirmed by the results of our studies on the example of mandarin grown in Georgia.

At the initial stage of the research, the main chemical parameters of the mandarin extract were determined. The data obtained are shown in table No. 1:

Table 1: *Physical-chemical parameters of mandarin residue*

№	The name of the indicator	Method	unit	Result
1	Soluble dry matter (TSS)	Refractometry	%	5,3±0,12
2	dry substance, by drying	by drying	%	19,5±1,75
3	Titrate acidity in terms of citric acid (pH 8.1)	titration	%	0,26±0,14
4	Vitamin C	HPLC	mg/%	38,80 ±0,2
5	Quantitative determination of flavonoids	spectral	%	2,24±0,4
6	total sugars, including:	HPLC	%	3,54±0,4
	glucose	HPLC	%	1,65±1,05
	fructose	HPLC	%	1, 73±0,34
	Sucrose	HPLC	%	0,16±0,0015
	Pectin substances, including:	spectral		
	total pectin	spectral	%	4,21±0,13

7	Hydropectin	spectral	%	1,60±0,036
	Protopectin	spectral	%	2,61±0,066
8	essential oils	titration	%	0,46±0,08
9	fiber	weight method	%	3,51±0,05
10	Quantitative determination of carotins	HPLC	mg/%	17,8±0,20

Table 1 shows that MP is characterized by a high content of biologically active substances: total flavonoids, carotenoids, vitamin C. MP contains much more antioxidant substances than pulp and juice. Among them, flavonoids are of great importance.

The data of the experiment conducted in order to intensify the process of extracting flavonoids from MP (acceleration of the process, increase in yield) are shown in Table 2:

Table 2: *The influence of the intensity of stirring of the extractant on the rate and yield of flavonoids in the extractant*

weed speed RPM	control	Duration of exposure, h				
		1	2	3	4	5
		Total amount of flavonoids,%				
355	3,92	2,16±0,07	3,41±0,12	4,47±0,17	4,48±0,13	4,4±0,15
500		2,64±0,08	4,47±0,15	4,47±0,17	4,48±0,14	4,42±0,17
710		2,59±0,07	4,48±0,16	4,48±0,18	4,46±0,17	4,39±0,18

The data of Table 2 show that in the control experiment - without the use of weeds, after the end of the process, the maximum concentration of the flavonoid complex is 3.92% based on dry weight. In the case of trial variants of the experiment, the maximum yield of flavonoids is 2.16-4.48%, which is achieved with various combinations of time and speed. Although the extraction yield at 710 rpm for 2 hours is high, foaming and disintegration of the crushed mandarin particles are observed, which ultimately degrades the quality of the flavonoids and subsequent processing. According to the results of the experiment, the speed of the weed was 500 rpm, and the time was 2 hours. The experiment shows that the use of an extractor equipped with a weed reduces the extraction time of the flavonoid substance from 24 hours to 2 hours and at the same time increases the yield by 12%. Based on the results of the research, a recommended technological scheme for the industrial production of flavonoids from mandarin juice production waste has been developed.

Identification and quantification of hesperidin

The study of flavonoids using a mass spectrometer - Hesperidin MW 610 Da, m / z (M-H-) 609, a fragment of the hesperidin 301 aglycone was identified in the mandarin extract and the preparation obtained from it, the results are presented on chromatograms (Fig. 1-4).

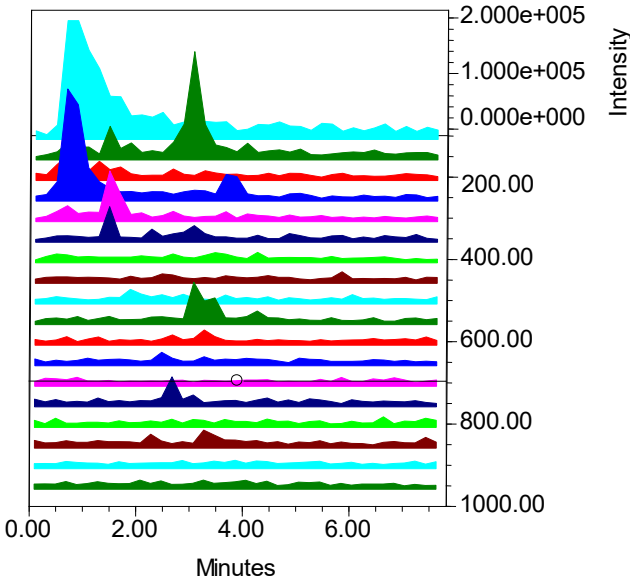

Figure 1: *Hesperidin in Mandarin Unshio residues UPLC-MS Chromatogram*

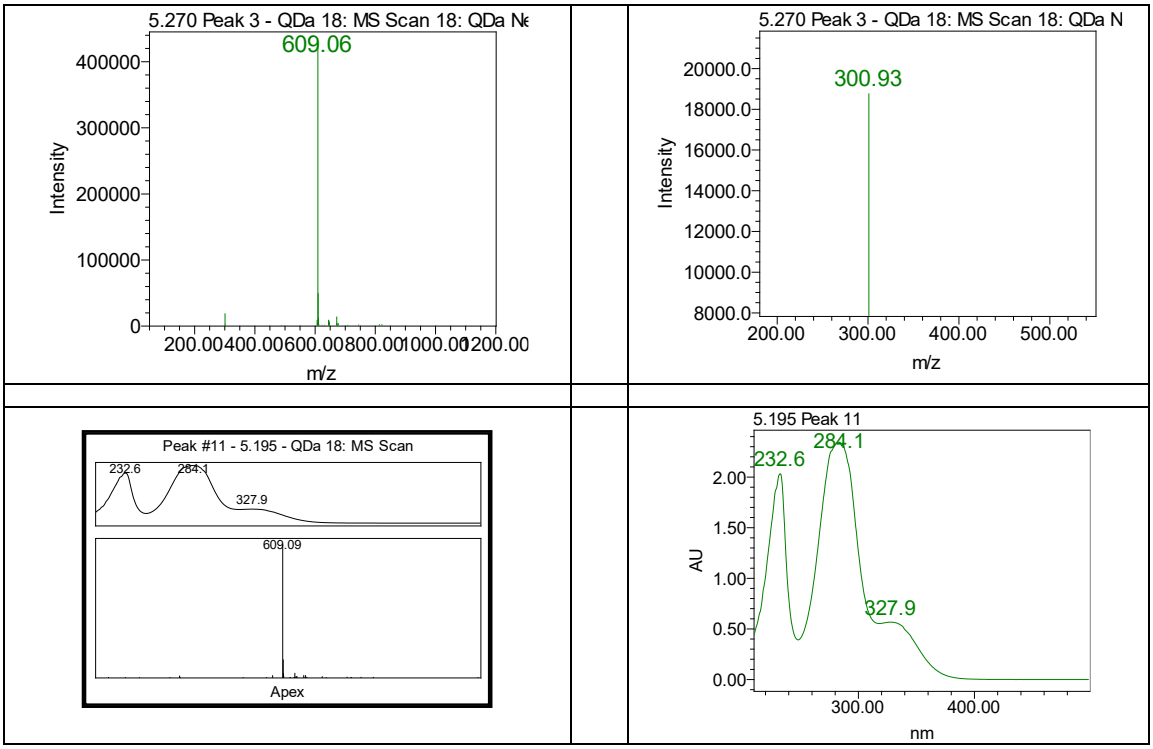

Figure 2: *Hesperidin obtained from PFE by recrystallization (ethanol + 20% DMSO), UPLC-MS spectrum SIR 609.09 Da, absorption max 284.1 nm.*

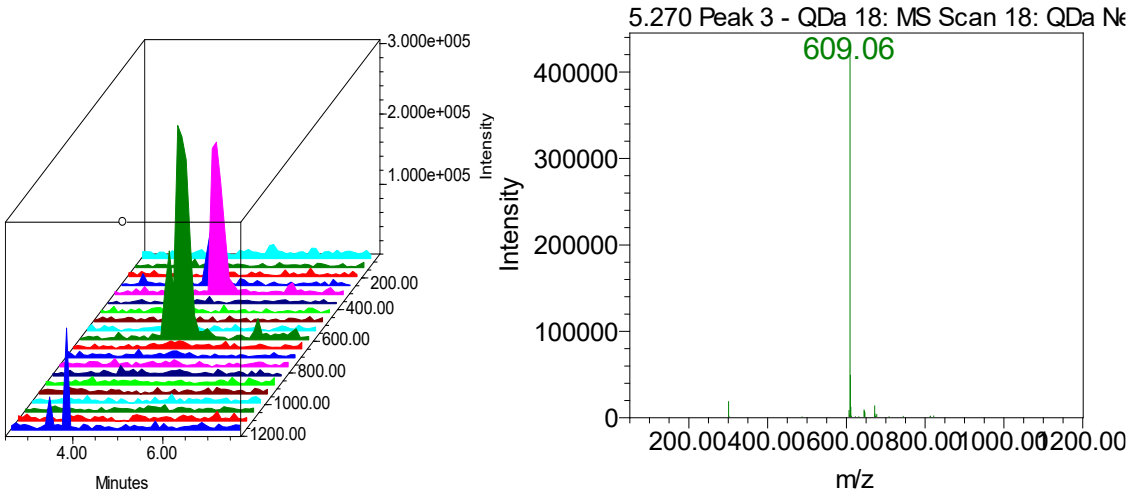

Figure 3: *Hesperidin obtained from PFE by recrystallization (NaOH+C2H5OH) UPLC-MS chromatogram, in 3D format, UPLC-MS spectrum SIR 609.09 Da*

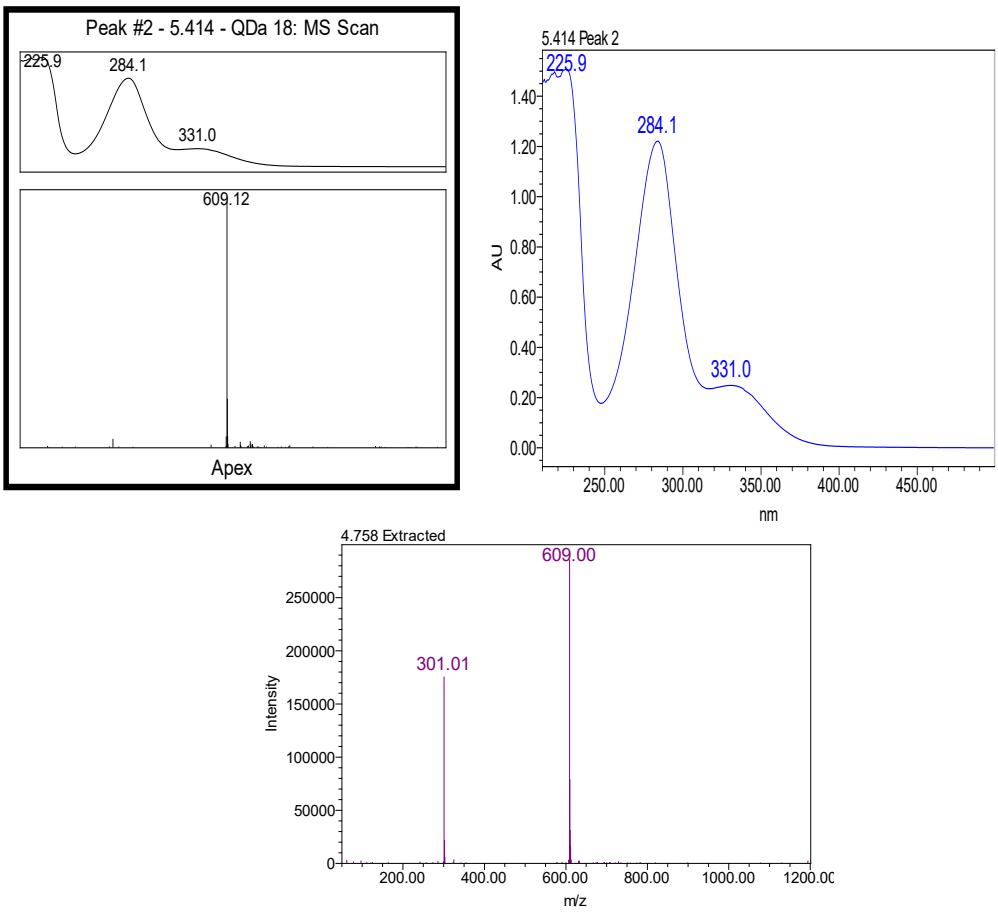

Figure 4: *UPLC-MS spectrum of standard hesperidin SIR 609.09 Da,absorption max 284.1 nm.*

Substances were identified using standard compounds (Fig. 4.) and mass of substances https://metlin.scripps.edu free database, as well as comparing the data of peer-reviewed literary publications.

To maximize the recovery of hesperidin, the samples have been eluted with different solvents, but extraction with a DMSO solution is optimal. The content of recrystallized hesperidin from the extract of unshiu flavonoids is 996 mg/g and with chromatographic extraction of 20% DMSO, a lower content of impurities is observed, the quantitative index of hesperidin in the resulting preparation is 90%.

Table 3: *Quantitative content of hesperidin in mandarin and preparations derived from it*

Sample name	mg/g
Hesperidin Standard	1,000.0
Hesperidin obtained from PFE, (ethanol + 20% DMSO)	983.0±39,32
Hesperidin obtained from PFE, ($NaOH+C_2H_5OH$)	996.0±39,84

The content of hesperidin in the mandarin peel is almost 1.5 times higher (0.750 mg/g) than in the whole cake; (0.496 mg/g). Recrystallization of hesperidin from PFE is effectively possible with NaOH+C2H5OH and ethanol + 20% DMSO. The results obtained are almost identical and allow them to be used for the recrystallization of hesperidin in an individual form from PFE.

IV. Conclusion

The chemical composition of oilcake, which remains during the processing of mandarin fruits (C. Unshiu), common in Georgia, has been studied, and it was found that it contains biologically active substances: flavonoids, carotenoids, vitamin C, pectin substances. The use of mandarin pomace to obtain PFE with a high content of hesperidin has been substantiated theoretically and experimentally. The kinetics of extraction of flavonoids and the factors, influencing it, have been determined; the optimal conditions for extraction and mixing were determined. Flavonoid content, hesperidin identification and quantitative content were determined by HPLC. Hesperidin was found to be the dominant flavonoid. There has been developed the method for the qualitative assessment of flavonoid extract, which makes it possible to achieve 90% hesperidin content in PFE.

Based on the results of the research, there has been developed a recommended rational technological scheme for the industrial production of flavonoids (hesperidin) from mandarin pomace, which ensures high yield and quality.

CONFLICT OF INTEREST.

The authors declare that they have no conflict of interest.

References

[1] Citrus peel as a source of functional ingredient: A reviewAuthor links open overlay panel Shafiya Rafiq[a] Rajkumari Kaul[a] S.A.Sofi[a] Nadia Bashir[a] Fiza Nazir[b] Gulzar Ahmad Nayik[c]
https://doi.org/10.1016/j.jssas.2016.07.006Get rights and content

[2] Ramana K.V.R., Govindarajan V.S., Ranganna S. 1981. Citrus fruits—varieties, chemistry, technology, and quality evaluation. Part I: Varieties, production, handling, and storage. Crit Rev Food Sci Nutr 15:353–431. Web of Science® Times Cited: 5

[3] Blauer R. Citrus: world markets and trade — USDA Foreign Agricultural Service, USDA Foreign Agricultural Service. 2016.
http://www.fas.usda.gov/data/citrus-world-markets-and-trade

[4] USDA: United States Department of Agriculture / Foreign Agricultural Service, 2010. Citrus: World Markets and Trade Available from: http://www.fas.usda.gov . Accessed 28.09.2010.

[5] Manthey J.A. , Grohmann K. Phenols in citrus peel byproducts. Concentrations of hydroxycinnamates and polymethoxylated flavones in citrus peel molasses. // J. Agric. Food Chem., 49 (7) (2001), pp. 3268-3273.

[6] Jayathilakan, K., Sultana, K., Radhakrishna, K. and Bawa, A.S. 2012. Utilization of Byproducts and Waste Materials from Meat, Poultry and Fish Processing Industries: A Review //. J. Food Sci. Technol., 49(3): 278-293

[7] Murakami, A., Nakumara, Y., Ohto, Y., Yano, M., Koshiba, T. and Koshimizu, K. 2000. Suppressive Effects of Citrus Fruit on Free Radical Generation and Nobelitin, an Anti-inflammatory Polymethoxy Flavonoids. // Biofactor., 12: 187-192

[8] Ajila, C. M., Bhat, S. G. and Rao, S. P. 2007. Valuable Components of Raw and Ripe Peels from Two Indian Mango Varieties. // Food Chem., 102(4): 1006-1011.

[9] Mouly P., Gaydou E.M., Auff ray A. (1998). Simultaneous separation of flavanone glycosides and polymethoxylated fl avones in citrus juices using liquid chromatography. // J. Chromatogr. 800: 171-179.

[10] Food and Nutrition Board, Institute of Medicine, 2001. Dietary reference intakes. Proposed definition of dietary fibre. A report of the panel on the definition of dietary fibre and the standing committee on the scientific evaluation of dietary reference intakes. National Academy Press, Washington, DC.

[11] Huet R. Les huiles essentielles d'agrumes. // Fruits. 1991, 4, 551-576.

[12] Espiard, E. Introduction à la transformation industrielle des fruits. (Ed) TEC &DOC. 2002. France: 259-265.

[13] Ramful, D.; Bahorunb, T.; Bourdonc, E.; Tarnusc, E.; Aruoma, O.I. Bioactive phenolics and antioxidant propensity of flavedo extracts of Mauritian Citrus fruits: potential prophylactic ingredients for functional foods application. // Toxicol. 2010, 278, 75-87.

[14] Bogucka –Kocka, A.; Woźniak, M.; Feldo, M.; Kocki, J.; Szewczyk, K., Diosmin – Isolation Techniques, Determination in Plant Material and Pharmaceutical Formulations, and Clinical Use. // Nat. Prod. Commun. 2013, https://doi.org/10.1177/1934578X1300800435

[15] Gong, Y.; Zhang, X.; L.; Yan, Q.; Yuan, F.; Gao, Y. Optimization of Subcritical Water Extraction Parameters of Antioxidant Polyphenols from Sea Buckthorn (Hippophaë Rhamnoides L.) Seed Residue. https://doi.org/10.1007/s13197-013-1115-7;

[16] Patience, N. A.; Schieppati, D.; Boffito, D. C. Continuous and Pulsed Ultrasound Pectin Extraction from Navel Orange Peels. // Ultrason Sonochem. 2021, 73, 105480. https://doi.org/10.1016/j.ultsonch.2021.105480;

[17] Wong-Paz, J. E., Aguilar-Zárate, P., Veana, F., Muñiz-Márquez, D. B. Impact of green extraction technologies to obtain bioactive compounds from citrus fruit residues. TIP Rev. Espec. En Cienc. Quím. - Biológicas 2020; pp. 1-11.

[18] Kirtadze N., Ardzenadze M., Bakuridze L., Berashvili D. Obtaining a substrate for P-vitamin activity from citrus processing residues. // Collection of Scientific Works of Tbilisi State Medical University Volume 50, Tbilisi 2016. pp. 74-76.

[19] Delia B. ,,Food Carotenoids Chemistry, Biology, and Technology" Rodriguez-Amaya University of Campinas, Universidade Federal da Fronteira Sul, Brazil, 2016.

[20] Evseeva O.S., Andreeva O.A. Development and validation of a method for the quantitative determination of flavonoids in some species of the genus citrus. Pyatigorsk Medical and Pharmaceutical Institute - a branch of the State Budgetary Educational Institution of Higher Professional Education of the Volgograd Medical University of the Ministry of Health of Russia

[21] Cortellino, G., Gobbi, S. and Torreggiani, D.2011. New Prospects for High Quality Ingredients Obtained from Citrus Fruit Peel. / 11th International Congress on Engineering and Food (ICEF). Procedia Food Sci., 1:1848-1853

[22] Wang Y.-C., Chuang Y.-C., Hsu H.-W. The flavonoid, carotenoid and pectin content in peels of citrus cultivated in Taiwan. // Food Chem. 106 (2008) 277-284.

[23] Ma Y-Q., Ye X-Q., Fang Z-X., Chen J-C., Xu G-H., Liu D-H. Phenolic Compounds and Antioxidant Activity of Extracts from Ultrasonic Treatment of Satsuma Mandarin (Citrus unshiu Marc.) Peels. // J. Agric. Food. Chem. 56 (2008) 5682-5690.

[24] Inga Kartsivadze; Aleko Kalandia Maia Vanidze; Merab Ardzenadze and Darejan Chikovani-The study of the content of Carbohydrates, Organic acids, Vitamin C in some varieties of Tangerine juice introduced in Ajara using High Pressure Liquid Chromatography- Vol. 8. No.4. 2019.©Copyright by CRDEEP Journals. All Rights Reserved.Contents available at: www.crdeepjournal.org // International Journal of Life Sciences (ISSN: 2277-193x) SJIF: 5.79

[25] Kvesitadze, G.I., Kalandiya, A.G., Papunidze, S.G., Vanidze, M.R. Identification and Quantification of Ascorbic Acid in Kiwi Fruits by High-Performance Liquid Chromatography. // Prikladnaya Biokhimiya i Mikrobiologiya, 2001, 37(2), pp. 246

[26] Amir Hossein Abbaszadeh. Pectin and galacturonic acid from citrus wastes" University of Borås School of Engineering SE-501 90 BORÅS, 2008.