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Four Valuable Bioactive Compounds Obtained from Citrus Waste Using Sequential Two-Step Ultrasound-Assisted Extraction Method

Mzia Tsitsagi[*], Imeda Rubashvili[*], Mariam Chkhaidze[*], Miranda Khachidze[*], Ketevan Ebralidze[*]

[*]*Petre Melikishvili Institute of Physical and Organic Chemistry, Ivane Javakhishvili Tbilisi State Univeristy, Tbilisi, Georgia*

(Presented by Academy Member Vladimer Tsitsishvili)

Citrus is one of the important agricultural crops in Georgia, and agro-industrial wastes of tangerine and orange juice concentrates and jams production present rich and promising sources of such valuable bioactive compounds as essential oil, carotenes, natural flavanone hesperidin and pectin, which could be applied by pharmaceutical and food industries. In the present research, two-step ultrasound-assisted extraction method for simultaneous obtaining of four natural products (essential oil containing D-limonene, pectin, hesperidin and beta-carotene) from citrus waste was developed using the concept of sequential stepwise technique. Under optimal conditions, the percentage of D-limonene in the extracted essential oil varies from 75% to 98%, the yield of pectin and hesperidin varies from 15% to 50% and from 60% to 80%, respectively, the recovered content of beta-carotene in the dried citrus waste materials varies from 25.6 μg/g to 29.9 μg/g for tangerine waste and 39.5 μg/g to 42.3 μg/g for orange waste. Hence, the sequence of the developed stepwise ultrasound-assisted extraction procedures is simple, effective, selective and low cost laboratory method which provides high quality of target products and can be used to develop a standard technological process for utilization of citrus wastes. *© 2021 Bull. Georg. Natl. Acad. Sci.*

Sequential extraction, citrus waste, ultrasound-assisted extraction

Citrus is the largest fruit crop in the world (100 million cubic tons per year). The important varieties cultivated commercially are orange, tangerine, lime, lemon and grapefruit. World production of oranges is projected to grow to reach 77 million tons in 2023, tangerine 28 million tons [1]. A high percentage of orange production (70 %) is used to manufacture derivative products and approximately 50-60 % of the processed fruit is transformed into citrus waste (peel, seeds and membrane residues). Annually, the citrus waste created by processing industries is estimated to be over 60 million tons worldwide. In order to prevent problems related to the disposal of this product and environmental concerns, this agricultural waste must be properly processed [2].

The agricultural waste is rich in sugars, fibers, organic acids, amino acids and proteins, minerals, essential oil (mainly D-limonene), lipids and large amounts of polyphenolic compounds and vitamins [3]. Citrus has been the most researched subject in the category of fruit crops in the last hundred years. In recent years, extraction techniques for the chemical, food and pharmaceutical industries have received a lot of attention because of the increase in energy prices, carbon dioxide (CO_2) emissions and other environment-related problems. Furthermore, tremendous growth in the production, processing and consumption of citrus fruits has created many challenges for researchers.

One of the most crucial topics in this regard is the development of methods using modern techniques to achieve the maximum recovery of the valuable compounds and by-products at a low cost [4]. In order to utilize citrus waste for industrial purpose to obtain many valuable bioactive compounds, it needs to cover the stable form. Citrus by-products are sensitive to biochemical and microbial degradations because of their high amount of moisture (70-80 %). Moreover, the bioactive compounds of citrus by-products could be submitted to enzymatic oxidation at different steps of processing. Citrus by-products stabilization is an essential step to facilitate the further uses (extractions of bioactive compounds for healthy products formulation). Dehydration at appropriate conditions, allows a decrease of moisture and water activity of the product and the inhibition of both oxidative enzymatic reaction and microorganisms growth allowing prolonging the shelf life of the product. Convective drying process is always used for drying of agro-food and by-products. It is less expensive than freeze-drying which is better appropriated to products with high benefit. The long exposition of the product to ambient temperature during convective drying could induce the degradation of thermo-sensitive compounds and antioxidant capacities of the product [5]. Modern concept focuses on complete utilization of agricultural wastes, when the mass reduces by 80% and use of eco-friendly separation technique requiring standard equipment for industrial application. Well-designed sequential extraction makes available selective and quantitative extraction of different valuable products, from one waste material [6-8].

In the present research, a simple and effective extraction procedure for valuable bioactive compounds from waste of the citrus fruits was developed using the concept of sequential stepwise technique. It has been observed that design of sequential stepwise extraction methodology requires to conduct the following preliminary studies: shelf life investigation and drying kinetics of wastes; selection of valuable target compounds available from agricultural wastes; selection of suitable extraction method from the following techniques - supercritical (SC) fluid - SC-CO_2 (SFE), ultrasound-assisted (UAE), magnetic stirring (MSE) and conventional extraction (CE); determining the sequence of steps for extraction of selected target compounds; selection of organic solvents; optimization of operational conditions; development and validation of simple, specific and precise analytical methods for identification and quantitative determination of extracted target products; evaluating general feasibility for large-scale production of these bioactive compounds and demand of local market.

Recently, it has been proposed a sequence of procedures for the extraction of target products from the tangerine peel including steps of extraction of essential oil by SC-CO_2, acetone-modified SC-CO_2 extraction of carotenes and methanol-modified SC-CO_2 extraction of flavanones, culminating in the recovery of hesperidin from extract and pectin from dry residue [7]. The proposed method provided high yields of target products, but for a number of small and medium-sized enterprises (SMEs) it turned out to be too difficult due to the use of sequential SC-CO_2 extraction method for large-scale production.

The present paper discribes an alternative, simple, low cost, sequantial stepwise extraction method for extraction of four valuable bioactive compounds – essential oil mostly composed of D-limonene ((4*R*)-1-methyl-4-prop-1-en-2-ylcyclohexene, $C_{10}H_{16}$), pectin, hesperidin and carotene pigment as beta-carotene from citrus waste. The developed method provides receiving more than one target product from one waste material.

Materials and Methods

The Test samples for extraction were tangerine and orange processing wastes as citrus waste. Tangerine waste material was provided by local juice manufacturer and orange waste material was obtained from fruit juice machine in laboratory conditions. The plant raw material was dried under the controlled environmental conditions (temperature - 30-40°C and relative humidity - 30-60 %) and protected from direct sunlight during 14 days.

The analytical standards of hesperidin ((2*S*)-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-[[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxy-2,3-dihydrochromen-4-one, $C_{28}H_{34}O_{15}$), galacturonic acid ((2*S*,3*R*,4*S*,5*R*,6*R*)-3,4,5,6-tetrahydroxyoxane-2-carboxylic acid, $C_6H_{10}O_7$) and beta-carotene (1,3,3-trimethyl-2-[(1*E*,3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*,17*E*)-3,7,12,16-tetramethyl-18-(2,6,6-trimethylcyclohexen-1-yl) octadeca-1,3,5,7,9,11,13,15,17-nonaenyl]cyclohexene, $C_{40}H_{56}$) were purchased from Sigma-Aldrich (Germany), as well as the HPLC/GC grade acetonitrile, methanol, ethanol, acetone, ethyl acetate, dimethyl formamide, dimethyl sulfoxide, 1,2-dichloromethane, *n*-hexane, chloroform and the analytical grade anhydrous sodium sulfate, acetic acid, hydrochloric acid, formic acid, acetic acid, sodium salt of ethylene diamine tetra acetic acid (EDTA), pectinase and phosphoric acid.

Water distillation was selected for extraction of essential oil from citrus waste. In order to isolate essential oil a sufficient quantity of water was added to plant material and brought to boil. The main characteristic of water distillation is that there is direct contact between boiling water and plant material, which makes a possibility of extraction of pectin. Citric acid was added for maintaining pH=1.5±0.1. Ethanol extraction procedure was used for the recovery of hesperidin and beta-carotene. Chloroform was used for separation of beta-carotene from hesperidin.

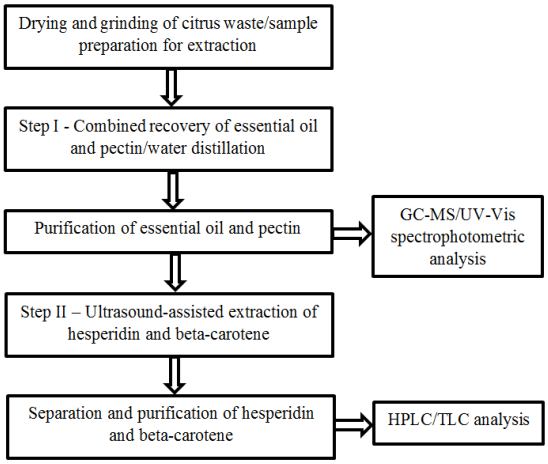
Dean Stark apparatus was used for simultaneous extraction of pectin and essential oil from citrus waste. 20 g of the dried and powdered citrus waste was weighed and placed in round-bottom flask, added 200 mL of distilled water. Citric acid was added for maintaining pH=1.5±0.1. The obtained mixture was heated to boiling point. The extraction was carried out during 1 h from the first drop of distillate until the amount of essential oil stabilized. Essential oil was separated from water layer, dried over anhydrous sodium sulfate, filtered by 0.45 µm membrane filter and stored in a refrigerator (2-8°C) for gas chromatography-mass spectrometry (GC-MS) analysis [7]. The obtained residue was dried and stored for further use. The hot acid extract was filtered through muslin cloth. The filtrate was cooled to the room temperature. Pectin-containing aqueous extract was coagulated by using an equal volume of ethanol at 4°C and was left for 1 h. The precipitate was recovered through centrifugation (4000 rpm for 15 min) and filtration, and then washed with 55 % and again with 70 % ethanol [7]. Finally, the dried and purified pectin was ground and kept for further analysis to determine total pectin using UV-Vis spectrophotometry (at wavelength 520 nm) [8].

The solid residue remained after the first step of extraction was dried, grounded again, weighed and immersed in beaker. Ethanol was added as extraction solvent for hesperidin and beta-carotene with a solid-liquid ratio of 1:10. Ultrasonic bath extraction was carried out with 20 kHz ultrasonic power, for 30 min at 30°C. Then the obtained mixture was filtered and concentrated. Syrup residue was diluted with a cold 6% acetic acid and allowed to stand for 6 h in order to crystallize. The precipitate was filtered through Buchner funnel and washed with chloroform. The crude hesperidin was heated with water at 70°C for 45 min., then filtered, dried and added again to chloroform. The white crystalline hesperidin was filtered through a Buchner funnel and dried. Melting point of the obtained purified hesperidin was 248-249°C; this result was complied with the value of the melting point of hesperidin analytical standard. The obtained pure hesperidin was identified by thin-layer chromatography (TLC) analysis using aluminum-backed silica gel 60 F_{254} plate (Sigma-Aldrich, Germany) with methanol/chloroform as mobile phase (R_f=0.72). The quantitative determination of hesperidin was carried out by high performance liquid chromatography (HPLC) with the validated analytical method. Beta-carotene containing filtrate was evaporated under reduced pressure and the obtained dried residue was analyzed using HPLC with the validated method [9]. The chromatographic analysis was performed using LC-20AD Prominence Shimadzu HPLC system (Japan) and GC-MS 790B1/5977A system (AG Technologies, USA) on the following columns: Agilent SB-C18 (4.6×250 mm, 5 µm) and HP-5 (30m×0.32mm×0.25µm), respectively.

The sequence of extraction procedures is illustrated in the scheme.

Results and Discussion

According to results of the GC-MS analysis carried out for determination of the composition of the extracted essential oil, D-limonene is a dominant contributor (75-98 %). The yield of essential oil obtained from orange waste was 0.7-1.7 %, in case of tangerine waste the percentage was 0.5-2.5 %. The composition and the yield of essential oil depend on the maturity of citrus.



Scheme. Sequential stepwise extraction.

The yield of pectin was varied from 15% to 50%, significantly depended on the type of citrus waste. The content of pectin was determined by external standard method of quantification using the analytical standard of galacturonic acid.

The recovered contents of beta-carotene in the dried citrus waste materials varied from 25.6 µg/g to 29.9 µg/g for tangerine waste and from 39.5 µg/g to 42.3 µg/g for orange waste. The percentage of beta-carotene in the dried powder varied from 75 to 89%.

The yield of hesperidin varied from 60% to 80% depended on the citrus waste material.

Conclusion

The most widely used method of utilization of agro-industrial wastes is dedicated to the recovery of only one valuable product from one waste material. However, this approach is not effective, because it cannot provide the waste minimization and value adding to the agro-industrial waste. Extraction of more than one valuable bioactive compound can significantly reduce volume and mass of waste

material. Unlike SF extraction, the polarity of organic solvents decreases in sequential extraction of essential oil, pectin, hesperidin and beta-carotene. Compared to sequential SF extraction of citrus waste, proposed method is simple in manipulation, selective, low cost and makes possible to extract simultaneously four different valuable compounds in two main steps from one waste material. Disadvantage of this method is the lower yield of beta-carotene in comparison with sequential SF-CO_2 extraction method, because of partial thermal degradation of carotene pigment due to relatively high temperature of essential oil hydro-distillation / pectin extraction step. Method is reproducible and convenient for large-scale replication [7].

Hence, the sequence of the developed stepwise ultrasound-assisted extraction procedures of four bioactive compounds from citrus waste material is simple, effective, selective and low cost laboratory method combined with the quality control UV-VIS spectrophotometric, HPLC and GC-MS quantitative validated analytical procedures, which provides high quality of target products and can be used to develop a standard technological process for utilization of citrus wastes.

ანალიზური ქიმია

ციტრუსის ნარჩენებიდან ოთხი ბიოლოგიურად აქტიური ღირებული ნივთიერების მიღება თანმიმდევრული ორსაფეხურიანი ულტრაბგერითი ექსტრაქციის მეთოდით

მ. ციცაგი*, ი. რუბაშვილი*, მ. ჩხაიძე*, მ. ხაჩიძე*, ქ. ებრალიძე*

*ივანე ჯავახიშვილის სახ. თბილისის სახელმწიფო უნივერსიტეტი, პეტრე მელიქიშვილის ფიზიკური და ორგანული ქიმიის ინსტიტუტი, თბილისი, საქართველო

(წარმოდგენილია აკადემიის წევრის ვ. ციციშვილის მიერ)

საქართველოში ციტრუსები მნიშვნელოვანი სასოფლო-სამეურნეო კულტურაა და მანდარინისა და ფორთოხლის წვენებისა და ჯემების წარმოების აგროინდუსტრიული ნარჩენები ისეთი ღირებული ბიოლოგიურად აქტიური ნივთიერებების მდიდარ და პერსპექტიულ წყაროს წარმოადგენს, როგორიცაა ეთერზეთი, კაროტინები, ბუნებრივი ფლავანონები – ჰესპერიდინი და პექტინი, რომლებიც შესაძლებელია გამოყენებულ იქნეს ფარმაცევტული და საკვები პროდუქტების წარმოებაში. წინამდებარე კვლევის ფარგლებში თანმიმდევრული საფეხურებრივი ტექნიკის კონცეფციის საფუძველზე შემუშავებულია ციტრუსების ნარჩენებიდან ორსაფეხურიანი ულტრაბგერითი ექსტრაქციის მეთოდი ერთროულად 4 ბუნებრივი პროდუქტის (D-ლიმონენის შემცველი ეთერზეთი, პექტინი, ჰესპერიდინი და ბეტა-კაროტინი) მისაღებად. ოპტიმალურ პირობებში ექსტრაჰირებულ ეთერზეთში D-ლიმონენის პროცენტული შემცველობა მერყეობს 75%-დან 98%-მდე, პექტინისა და ჰესპერიდინის გამოსავლიანობა შეადგენს შესაბამისად 15%-დან 50%-მდე და 60%-დან 80%-მდე; აღდგენილი ბეტა-კაროტინის შემცველობა ციტრუსის ნარჩენების მშრალ მასაში მერყეობს 25,6 მკგ/გ-დან 29,9 მკგ/გ-მდე მანდარინის ნარჩენის შემთხვევაში, ხოლო 39,5 მკგ/გ-დან 42,3 მკგ/გ-მდე ფორთოხლის შემთხვევაში. ამრიგად, თანმიმდევრული საფეხურებრივი ულტრაბგერითი ექსტრაქციის შემუშავებული პროცედურების ერთობლიობა წარმოადგენს მარტივ, ეფექტურ, სელექტურ და დაბალბიუჯეტიან ლაბორატორიულ მეთოდს, რომელიც შესაძლებლობას იძლევა მიღებულ იქნეს მაღალი ხარისხის სამიზნე ნივთიერებები და შემუშავდეს სტანდარტული ტექნოლოგიური პროცესი ციტრუსების ნარჩენების უტილიზაციის მიზნით.

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