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EXTRACTION AND ANALYSIS OF OLEANOLIC ACID AND URSOLIC ACID FROM APPLE PROCESSING WASTE MATERIALS USING ULTRASOUND-ASSISTED EXTRACTION TECHNIQUE COMBINED WITH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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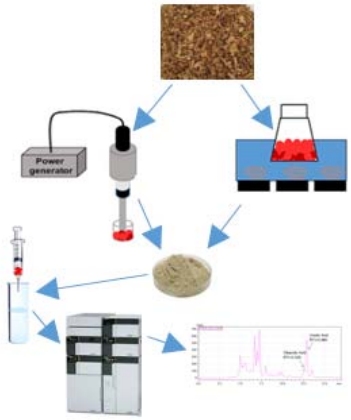
The aim of the present study was to develop a simple, effective, eco-friendly, reproducible and high-yield two-stage ultrasound-assisted extraction (UAE) procedure combined with quantitative determination high performance liquid chromatographic (HPLC) method for obtaining isomeric triterpene acids – oleanolic acid (OA) and ursolic acid (UA) in the crystalline dried powdered form from apple processing agro-industrial waste material. A rapid, sensitive and specific HPLC method was developed and validated with respect to robustness, specificity, linearity-range, accuracy, precision and sensitivity. The effect of the nature and the volume of the extraction solvent, the extraction time and the sample size on the extraction efficiency were investigated. The optimal conditions for high-yield extraction were found.



INTRODUCTION

In the recent years, one group of natural products called pentacyclic triterpenoids has attracted a lot of attention due to its unique and strong biological and pharmacological activities.[1] The main pentacyclic triterpenes namely isomeric triterpene acids – oleanolic acid (OA) and ursolic acid (UA) are found in plant kingdom, many medicinal herbs, fruits and vegetables as the free acid or aglycones. The above-mentioned compounds have low toxicity and a wide variety of reported and approved pharmacological activities, including anticancer, chemopreventive, hepatoprotective, antiviral, antibacterial, anti-inflammatory, anticardiovascular, antiatherosclerostic, antidiabetic, antioxidant, immunomodulatory and gastroprotective properties.[2–14] OA and UA are also utilized in the preparation of food supplements[15] and important ingredients of cosmetic formulations[16] and sport supplements.[17] It has been reported that UA can stimulate muscle growth and enhance the epidermal permeability barrier recovery in the skin.[18] The chemical structures are given in Figure 1.

Due to the wide range of applications, these bioactive triterpenoid compounds have a high commercial value. Therefore, the efficient and high-yield extraction to obtain these bioactive compounds from raw materials has a great significance and practicability.

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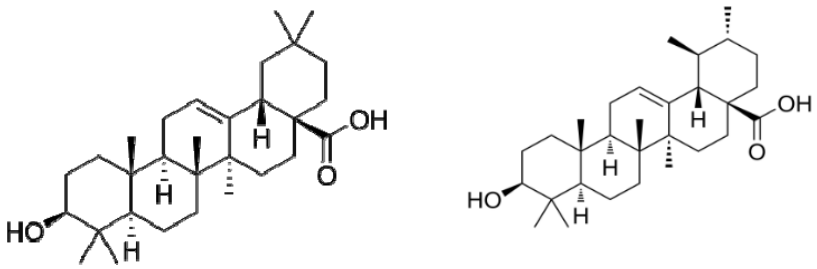

(a) (b)

Fig. 1 – The chemical structures of oleanolic acid (a) and ursolic acid (b).

Apple pomace as the waste material of the apple processing industry (the residue left after juice extraction) represents approximately 25% of the processed apple and a low-cost, rich source of fruit-derived bioactive compounds with valuable properties including OA and UA. Apple pomace consists of apple peels, seed, and stem with approximate composition of 95%, 2–4%, and 1%, respectively.[19–20] The surface of apple is covered by a lipophilic layer called cuticle having two main components: the first component containing compounds that cannot be extracted by solvents due to their polyester-type biopolymer structure mainly composed of fatty acids and the second component containing compounds with a variety of hydrocarbon chains or conjugated rings, including pentacyclic triterpenes released by solvent extraction.[21] Therefore, extraction of these bioactive compounds can be achieved and used for the development of functional foods.

Extraction is the essential step in the recovery and purification of target compounds from raw materials. There is no universal extraction technique for the isolation of various types of compounds from the sample. Therefore, an important issue is to verify different conventional and modern techniques for their efficiency towards target compounds. The selection of suitable extraction technique for obtaining target bioactive substances from agro-industrial waste materials depends on the nature and physical-chemical properties of the compounds, the quality of extracts and the yield; as well as on the conditions, the existence of appropriate analytical method of quantitative determination and economic expediency of the laboratory process. The technique should be reproducible, fast, simple, inexpensive and eco-friendly as possible. Due to the complex composition and the presence of interfering compounds in the sample, a great deal of research should be carried out to develop and validate an effective, sensitive and specific analytical method for quantitative estimation of target bioactive compounds.

Conventional extraction techniques, such as Soxhlet extraction,[22–25] reflux extraction[26–29] and maceration[30–32] have been used to extract different triterpenes and their derivatives, including OA and UA from plant materials. However, the extraction methods are solvent-, time, and energy-consuming. They are characterized by low yield and the loss or degradation of target compounds. The consumption of the large volumes of organic solvents is harmful to human health and the environment. Besides, the use of organic solvents in the food, cosmetic and pharmaceutical industries has many restrictions. In contrast, recently, the modern extraction methods namely supercritical fluid (SFE), microwave-assisted (MAE) and ultrasound-assisted extraction (UAE) are successfully used as the best eco-friendly techniques, with many advantages such as high extraction efficiency, expeditiousness, good reproducibility, possible recycling, higher speed and low consumption of solvents, energy and time. In recent years, many reports have been published on the application of SFE, UAE and MAE for the extraction of target compounds from different raw materials.[19,33-45] However, it is unknown whether the extraction efficiencies of OA and UA from apple processing waste materials could be improved by UAE.

As for the issue of quantitative estimation of OA and UA, HPLC has emerged as a popular analytical technique for quantitative determination of these compounds from plant materials. Several publications have reported HPLC analyses of OA and UA obtained from various types of biomass.[46,47]

A review of the literature revealed that none of any articles will be obtained where the UAE technique combined with the HPLC procedure for obtaining and determining quantitatively OA and UA from apple processing waste materials would

be discussed, also the analytical procedures are not appropriate for our analytical purposes.

The aim of this study was to develop and validate a new, selective, reproducible and high-yield extraction method by ultrasound-assisted technique for obtaining OA and UA from apple pomace as waste materials of apple processing industries and an effective, specific, sensitive and rapid HPLC analytical procedure to determine quantitatively these target compounds.

EXPERIMENTAL

Apple pomace as apple processing waste material was provided by local apple fruit manufacturer. The raw material was dried in laboratory room under the controlled conditions (the temperature – 20–25ºC and the relative humidity – 30–60%) and protected from direct sun light. The sample was ground manually to be powdered and stored in refrigerator before extraction.

The certified analytical standards of OA and UA, the HPLC grade acetonitrile and methanol, the analytical grade potassium dihydrogen phosphate, sodium hydroxide, hydrochloride acid, anhydrous formic acid, absolute ethanol and 2-propanol were purchased from Sigma-Aldrich (Germany).

The HPLC grade purified water was prepared using Milli Q Adventage A10 purification system (France). Two types of sonication – Jy92-Iidn ultrasonic homogenizer (probe-type) (China) and dual-frequency ultrasonic bath DW-5200DTS (bath-type) (China) were used for UAE.

The ultrasound frequency was 25 kHz; the temperature was controlled at 25±2ºC during ultrasonication; ethanol and 2-propanol were selected as non-toxic and the best extraction solvents for triterpene acids based on the reports.[48-50] Seen from the chemical structures of OA and UA, they have relative low polarities, and 2-propanol and ethanol polarities will significantly contribute to the effectiveness of the extraction. The two stage UAE was carried out by adding 5–20 g of the powdered dried sample and 50-200 mL of solvent in 200 mL extraction vessel equipped with digital temperature controller for 10-40 minutes. After both extraction stages, the crude extract solutions were centrifuged at 4000 rpm for 10 minutes, and then the obtained supernatants were collected to evaporate under air flow for removing organic solvent. Then 50 mL of purified water was added to the obtained wet powder containing OA and UA and mixed vigorously for a few minutes. In order to remove water soluble impurities, the obtained suspension was heated at 50ºC for 30 minutes, and then centrifuged at 4000 rpm for 10 minutes and the precipitate was dried. In order to remove non-polar impurities, *n*-hexane was added to the dried powder and the obtained suspension was stirred for 1 hour, and then centrifuged at 4000 rpm for 10 minutes. The precipitate was dried, and then dissolved in hot alkaline ethanol – a mixture ethanol and strong sodium hydroxide solution 90:10 v/v (pH~10). Then the pH value of this solution was adjusted to 7.0±0.05 with hydrochloride acid solution and the obtained solution was allowed to stand for 24 hours. The obtained crystalline solid was separated from solution through centrifugation, and then dried under air flow to obtain an extracted product. The scheme UAE combined with HPLC analysis is shown in Figure 2. The UAE methods for obtaining other valuable natural bioactive compounds were developed and validated in the previous reports by the authors.[51-53]

The chromatographic analysis was performed using LC-20AD Prominence Shimadzu HPLC System (Japan). Analytical balance ALX-210 (USA) and pH-meter Hanna Instruments HI 2211 (USA) were used for preparation of solutions. All the measuring equipment was appropriately calibrated. The experiment was carried out in controlled area (temperature, t=22±3°C, relative humidity, RH=45±15%).

The analytical method was developed using the HPLC column – Agilent SB-C18 4.6×250 mm, 5 µm (USA) with an isocratic elution of mobile phase (MP) containing a mixture of phosphate buffer solution pH 6.0 (6.8 g/L potassium dihydrogen phosphate solution adjusted to pH 6.0 with strong sodium hydroxide solution), acetonitrile and methanol (20:30:50 v/v) filtered through PVDF 0.45 µm membrane filters and degassed; The flow rate of mobile phase was 1.0 mL/min; The UV-spectrophotometric detection was performed at the wavelength – 210 nm; The injected volume was 20 µL; The column temperature was maintained at 35°C. The analytical data was reported using HPLC system software.

The developed HPLC method was validated with respect to the following validation parameters: standard solution stability study, system suitability test (SST), specificity, linearity-range, precision, accuracy and sensitivity according to ICH Q(2) guideline[54] and the appropriate methodologies reported by the authors.[51-53,55-57] Microsoft Excel 2016 was used for statistical assessment and regression analysis.

The analytical standard of OA/UA diluted in a mixture of anhydrous formic acid and methanol 2:98 v/v (diluent) was used as the standard solution at the concentration – 0.25 mg/mL. The both standard solutions were mixed 1:1 v/v and the obtained solution was used as the resolution check solution at the concentration – 0.125 mg/mL of each analyte. For preparation of a test solution, the dried extracted product was transferred to 10 mL volumetric flask and diluted to volume with the diluent, mixed well. The obtained solution was filtered through 0.45 µm PVDF microporous membrane filter. Additionally, the spiked test solution was prepared according to the following way: 1-1 mL of the standard solution of OA and UA were transferred to 20 mL volumetric flask and added 1 mL of the test solution, then diluted to volume with the diluent and mixed well.

The concentration of OA/OA – Cu, mg/mL in the test solution was calculated by the following formula:

$$Cu = Au \times W_1 \times D \times P / As \times 100 \quad (1)$$

where, Au – Peak area of OA/UA obtained with the test solution; As – Peak area of OA/UA obtained with the standard solution; W_1 – Weight of OA/UA analytical standard, mg; D – The dilution factor, mL; P – Purity of analytical standard, %.

The content of OA/UA – X (the extraction yield), mg per 1 g of the dried sample of raw material (apple processing waste material) was calculated by the formula:

$$X = Cu \times V / W_2 \quad (2)$$

where, Cu – the determined concentration of OA/UA in the test solution; V - The dilution volume of the dried sample, mL; W_2 – Weight of the dried sample, g.

In order to optimize the selected extraction conditions and to establish the optimal extraction parameters, the design of experiments (DoE) was used; the quantitative and qualitative critical parameters of UAE were considered and the five parameters (Xi) with two levels ("+" and "–") were selected which are summarized in Table 1. The extraction yield, mg/g of each target compound was used as the response for assessment of the UAE procedure. The experiments were conducted in 2^{5-2}= 8 runs for five two-level factors.

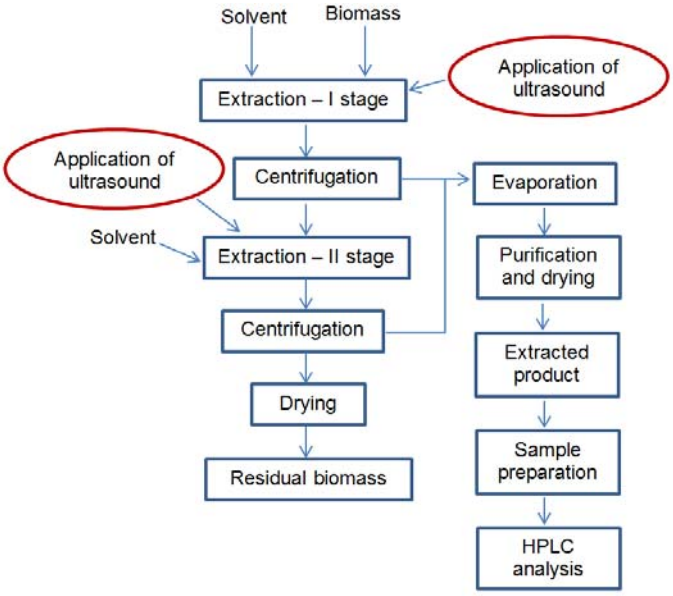

Fig. 2 – The scheme of UAE combined HPLC analysis.

Table 1

Design of experiments for UAE procedure

Name of extraction parameter - Xi	**Unit**	**Level**	
		–	+
The volume of solvent – X1	mL	50	100
Solvent on the stage I and II – X2	–	2-Propanol/ethanol	Ethanol/ethanol
The sample size – X3	g	10	20
The concentration of ethanol – X4	%	95	100
The extraction time – X5	min	20	30

RESULTS AND DISCUSSION

Ultrasound-assisted extraction

The results obtained with the extraction process indicate that the effects of the extraction time, the volume of extraction solvent and the sample size of raw material on the extraction efficiency are significant for both target compounds. The effect of the extraction time (t) was investigated by carrying out the experiments at 10, 20, 30 and 40 min; other extraction parameters were set as follows: the ultrasound was set at comparatively lower ultrasonic power – 25 kHz; the sample size – 10 g; the extraction volume of solvent – 100 mL, 2-propanol and 95% ethanol were used as extraction solvents for the extraction stage I and II, respectively; the temperature was 25±2ºC. The results are displayed in fig. 3. It was observed that the extraction yields of OA and UA were characterized by the similar trends at the same extraction conditions, but the extraction yield of UA was higher than that OA. The extraction yield increased exponentially from 10 to 20 minutes, and then decreased, when the ultrasonication time was longer than 10 minutes. Most of OA and UA were extracted during the 1/2 of total extraction time (40 min). The maximum yields of OA and UA were 1.535 mg/g and 4.585 mg/g, respectively, which were obtained at 20 min. The results show that ultrasound could accelerate the establishment of equilibrium for dissolving of target compounds between biomass and the extraction solvent in a short time. A long exposure to ultrasonication causes a decrease in the extraction yields of both compounds; therefore, ultrasound degradation leads to the reduction of the amount of OA/UA due to the side effect of ultrasound. The optimal extraction time could be considered 20 min.

The effect of the solvent volume (V) on the extraction yield (X) was investigated by carrying out the experiments using 50, 75, 100 and 125 mL of solvent for both extraction stages, 2-propanol and 95% ethanol were used for the extraction stage I and II, respectively. Other extraction parameters were set as follows: the ultrasonic power –25 kHz; the sample size – 10 g; the extraction time – 20 min; the temperature was 25±2ºC. The results

shown in fig. 4 indicate a slightly different behavior between OA and UA extraction yields. The extraction yield increased when the solvent volume was from 50 mL to 100 mL and the yield decreased from 100 to 200 mL. The maximum yield of OA and UA was obtained at 100 mL. The volume of the extraction solvent should be sufficient and appropriate to dissolve more effectively components, reach equilibrium of isolation of the target bioactive compounds from biomass in a short time and to enhance the extraction yield. A further increase and larger volume of the extraction solvent significantly reduce the yield; this was observed especially, in the case of UA. There was an increase in the yield of OA when the volume of solvent was from 50 to 100 mL, after 100 mL the yield became almost constant, OA yield did not change during the variation of the volume of solvent. This is probably due to the fact that the yield of OA is relatively small compared to the yield of UA; OA is more stable during extraction by ultrasonication. Based on these results, 100 mL was considered as optimal volume of the extraction solvent.

To investigate the effect of the sample size (W_2) on OA and UA extraction efficiency, the experiments carried out by using 5, 10, 15, 20 g of samples in different extraction times – 20 min and 30 min. The results show that the yield of both target compounds depends on the sample size, by itself, this parameter varies according to the extraction time, and a similar trend is observed in the case of both compounds (Fig. 5). The highest extraction yield was observed when the sample size was 10 g in case of the extraction time was 20 min; when the extraction time was 30 min, the maximal yield was achieved at 15 g of sample size. The prolonged extraction time to 30 min for larger sample size had no significant effect on the extraction efficiency. The results confirmed that the effect of sample size on the extraction yield is a function of the extraction time. The less the sample size is, the less is the extraction time and the more the sample size is, the more is the volume of solvent and the extraction time. The ultrasonication was conducted by both ultrasound techniques – ultrasound probe and ultrasound bath. The percentage differences between the extraction yields of OA and UA obtained with two different ultrasound techniques do not exceed 5.0%. Both ultrasonic techniques are suitable to carry out UAE with the high extraction efficiency.

In order to determine all the optimal extraction parameters, 8-run experiments were carried out according the DoE (Table 1). The extraction yield as the variable was used to establish optimal conditions for the developed UAE procedure. The results of 8-run experiments are given in Table 2. The experiments results revealed that there was another important factor – the concentration of ethanol affected on the extraction process. The yields of OA and UA simultaneously reached highest values at 95% of the ethanol concentration; therefore, 95% ethanol is suitable solvent for the extraction procedure. The maximal extraction yields of OA and UA – 1.535 mg/g and 4.585 mg/g, respectively achieved at the following extraction parameters: the ultrasound frequency – 25 kHz; the sample size – 10 g; the extraction volume of solvent – 100 mL, 2-propanol and 95% ethanol were used for the extraction stage I and II, respectively; the temperature was 25±2ºC. These extraction parameters were established as the optimal conditions for two-stage UAE procedure for obtaining OA and UA from apple processing agro-industrial materials.

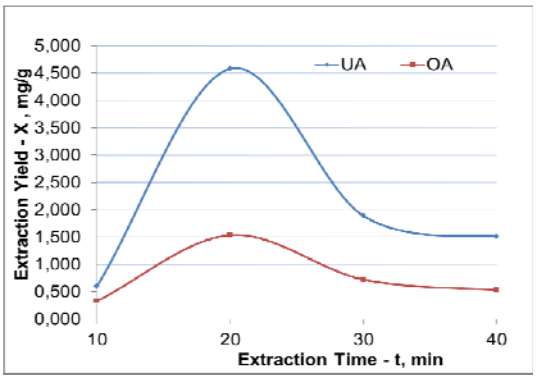

Fig. 3 – The effect of the extraction time (t) on the extraction yield (X) of OA and UA.

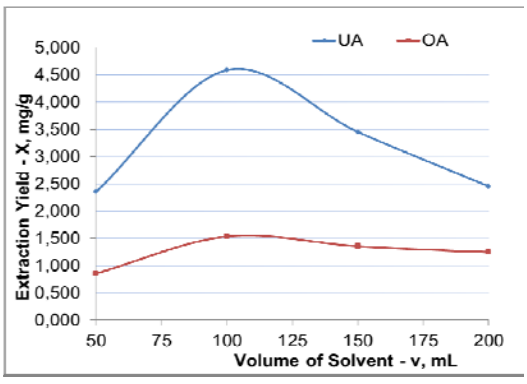

Fig. 4 – The effect of the volume of extraction solvent (V) on the extraction yield (X) of OA and UA.

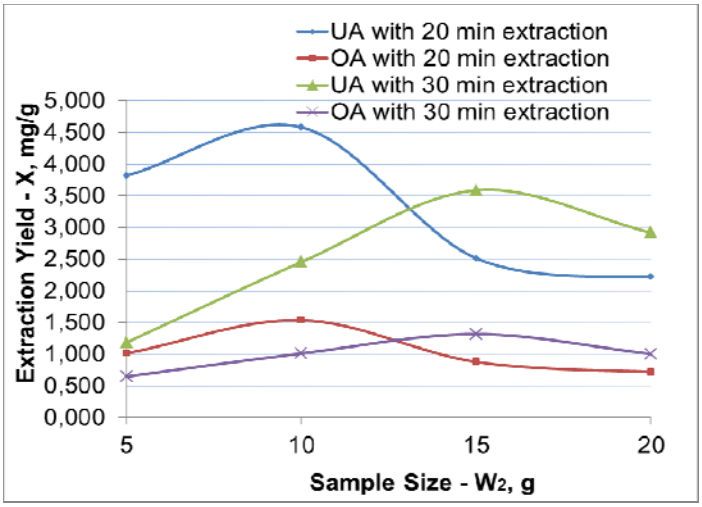

Fig. 5 – The effect of the sample size (W_2) on the extraction yield (X) of OA and UA in different extraction times.

Table 2

The results of 8-run experiments

Experiment number	Parameters - Xi					Extraction yield, mg/g	
	X1	X2	X3	X4	X5	OA	UA
1	+	+	+	+	+	0.541	2.614
2	+	+	-	+	+	0.798	2.412
3	+	-	+	-	+	1.022	4.114
4	+	-	-	-	-	1.535	4.585
5	-	+	+	-	-	0.856	3.482
6	-	+	-	-	+	0.962	3.144
7	-	-	+	+	-	0.412	2.633
8	-	-	-	+	+	0.641	2.731

Analytical method validation

The final chromatographic conditions were determined by optimizing the system parameters: the wavelength for detection, the composition of MP, the flow rate, the column and the injection volume. The SST parameters: theoretical plates, tailing factor and resolution between the principal peaks were checked.

The specificity test was checked by injecting the standard solution, the resolution check solution, the spiked test solution and the background control – blank (diluent) solution. The specificity test results have shown that there is no interference from the diluent/secondary peaks at the retention time (RT) of each analyte peak. The OA and UA peaks were pure and purity factors were more than purity threshold (990.0). Figure 6 show the chromatograms obtained from the standard solution, the resolution check solution, the spiked test solution and the blank solution, respectively. The sensitivity of the method was determined with respect to the limit and quantitation (LOQ).

In order to study the linearity-range, the working standard solutions were prepared at eight different concentration levels (the concentration range was 0.0001–0.5 mg/mL for UA and 0.000075–0.5 mg/mL for OA) and injected by six replicates (n=6) for each concentration level. The linearity was checked by the square of correlation coefficient – R^2 (acceptance criteria: >0.998), the relative standard deviation of peak areas – RSD_A (acceptance criteria: <2.0%) at all concentration levels excluding the last concentration level which should not be more than 10%, the RSD of retention times – RSD_{RT} (acceptance criteria: <1.0%) and the percentage slope (acceptance criteria: <10.0%). The last concentration level was estimated as the LOQ; the signal-to-noise – s/N for the LOQ should be not less than 10.

The determined LOQ for OA and UA are presented in Table 3. The calibration curves (linearity graphs) were constructed by plotting the average peak areas against the corresponding concentrations of the injected working standard solutions that indicate a perfect linearity for each

compound. Fig. 7(a) and 7(b) show the calibration curves for OA and UA, respectively.

The chromatographic system performance was checked in each validation parameter study. The SST was performed by using six replicate injections (n=6) of the standard solution of both compounds at the concentration – 0.25 mg/mL. The following parameters – the RSD_A, the RSD_{RT}, the peak tailing factor (the USP coefficient of the peak symmetry $S=W_{0.05}/2f$), the column efficiency – the number of theoretical plates and the resolution factor between UA and OA were measured. The results are summarized in Table 4.

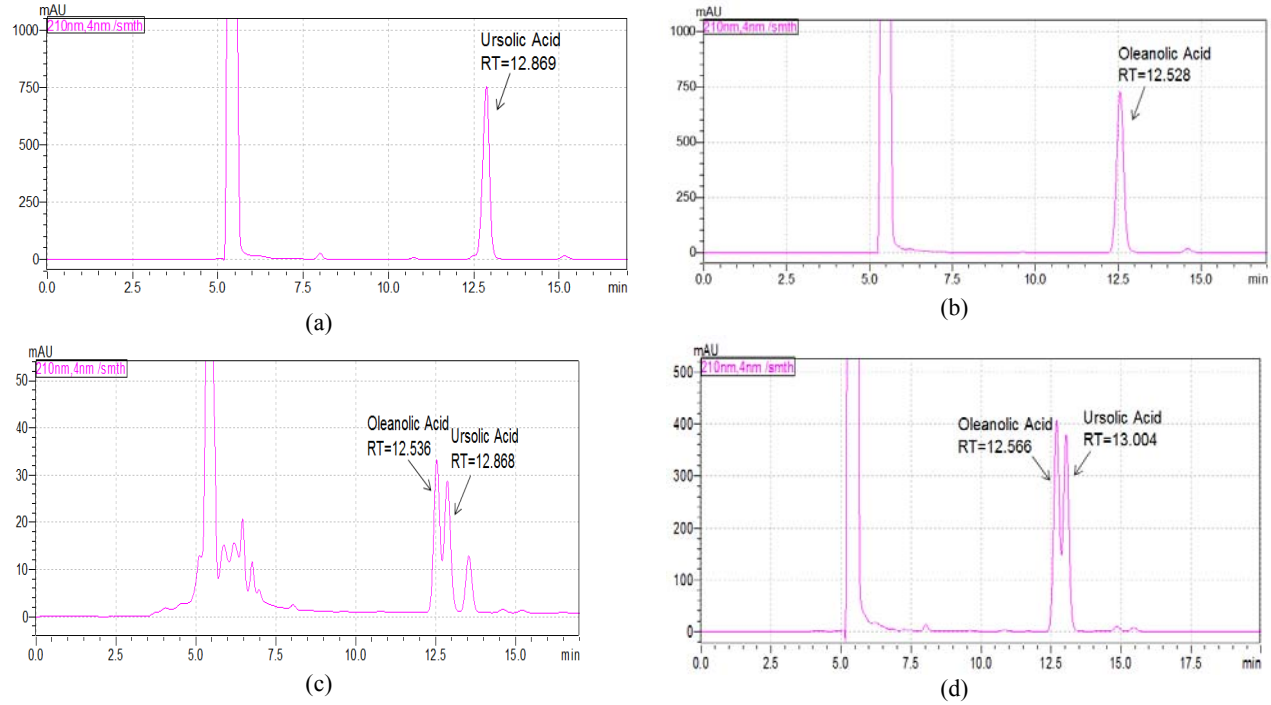

Fig. 6 – The chromatograms of the OA standard solution (a), the UA standard solution (b), the spiked test solution (c) and the resolution check solution (d) detected on 210 nm.

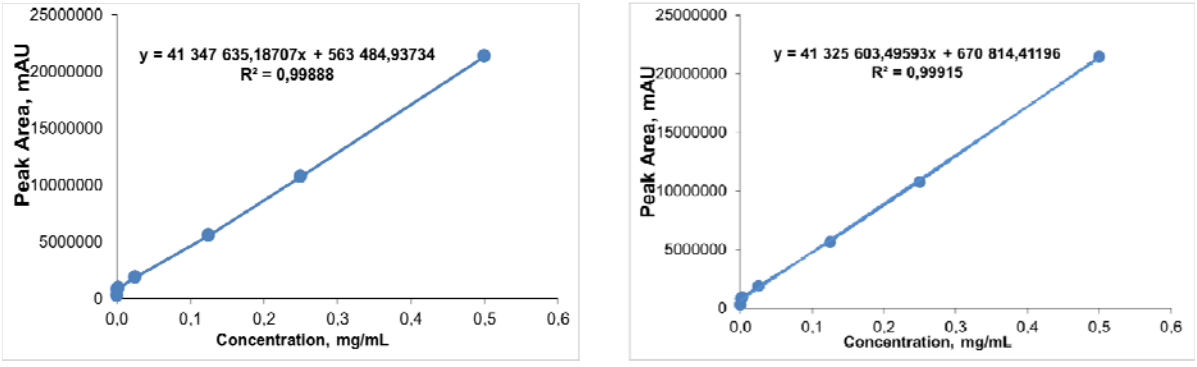

Fig. 7 – The calibration curves for OA (a) and UA (b).

Table 3

The LOQ of the HPLC method

Parameter	Value	
	OA	UA
LOQ, mg /mL	0.000075	0.0001
RSD_A for LOQ (n=6)	6.001	7.343
RSD_{RT} for LOQ (n=6)	0.050	0.073
s/N	21.5	11.4

Table 4

The system suitability test parameters results

SST Parameter	OA	UA	Acceptance criteria
Column efficiency	>16 668	>18 457	>2000
RSD_A (n=6)	0.543%	0.697%	<2.0%
RSD_{RT} (n=6)	0.158%	0.185%	<1.0%
Tailing factor (USP symmetry)	0.99	0.97	0.8-1.5
Resolution factor	0.85	>0.75	

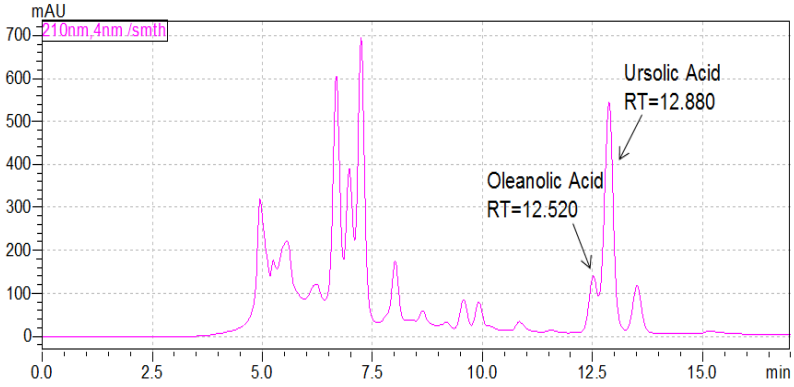

Fig. 8 – The chromatogram obtained with the test solution detected at 210 nm.

The precision of an analytical method was estimated by measuring repeatability (intra-day precision) on six individual determinations of each analyte in the test solution at the same concentration. The repeatability was checked by the RSD_A (acceptance criteria: ≤3%), the RSD_{RT} (acceptance criteria: ≤1%) and the RSD of determined concentrations (mg/mL) for six individual determinations of each target compound – OA/UA which should not be more than 5.0%. The chromatogram obtained with the test solution is shown in Figure 8. The precision study results show that the values of RSD_A, RSD_{RT} and RSD, % of determined concentrations (mg/mL) for each analyte comply with acceptance criteria; the RSD, % of determined concentrations (mg/mL) of OA and UA are 3.170% and 4.866%, respectively. The obtained results indicate that this HPLC method has a good precision and the developed extraction procedure allows us to obtain the target compounds with high reproducibility.

The accuracy of the method was assessed by comparing the analyte amount determined versus the known amount spiked at three different concentration levels (80, 100, 120% of each standard solution concentration) with three replicate injections (n=3). The test solutions were spiked with the standard solution at 0.25 mg/mL concentration. The accuracy is expressed as the percentage of standard compound recovered from the spiked test solution (test solution+standard solution) with a corresponding RSD,%. The average recovery rates for each concentration level of the spiked test solution and the main recovery should be within 95.0–105.0%, also the RSD of the percentage recoveries (n=3) should be <5.0% (acceptance criteria).

The recovery – Rec, % for each concentration level of the spiked test solution was calculated by the following formula:

$$\text{Rec, \%} = (Au_1 - Au_2) \times D \times 100 / As \qquad (3)$$

where, Au_1 – Peak area of OA/UA obtained with the spiked test solution (endogenous added OA/UA standard solution); Au_2 – Peak area of OA/UA obtained with the test solution; As – Peak area of OA/UA obtained with the standard solution; D – Dilution factor. The results of the recovery are given in Table 5 which are within accepted limits indicating the accuracy of the method.

The standard solution stability was studied initially, after 24 hours, 3, 5, 7 days stored under refrigeration against the freshly prepared standard solution. The stability was checked using two standard solutions and by the percentage difference between peak areas of standard solution stored and freshly prepared one which should not be more than 5.0% (acceptance criteria). The bias in terms of peak area between two standard solutions should be within 0.98–1.02 (acceptance criteria). The percentage difference between peak areas obtained with two standard solutions, one stored under refrigeration for 7 days and another prepared freshly is 2.35% and 4.51% for OA and UA, respectively.

Table 5

The accuracy results

Compound	Recovery rate, %			RSD, % (n=3)	The average recovery, %	The main recovery, %
	80%	100%	120%			
OA	96.32	97.54	96.69	1.75	96.85	96.90
UA	96.65	98.19	95.97	0.53	96.94	

Estimation of OA and UA content in apple processing waste materials

The content of OA and UA in the extracted product obtained with the developed two-stage UAE procedure in optimal conditions was estimated. The contents of the target bioactive compounds expressed in mg per 1 g of the dried sample of raw material obtained from apple processing wastes were calculated. The results indicate that the content of OA and UA, mg/g in apple processing waste materials varies from 1.355 to 1.544 mg/g (from 0.136% to 0.154%) and from 4.289 mg/g to 4.645 mg/g (from 0.429% to 0.465%), respectively; the total content of these triterpenoid acids in the obtained extracted product is high and varies from 56.72 % to 61.29 %; The average value of the total contents of OA and UA equals to 59.12%. These findings suggest that the optimum quantities of these compounds have been reached in the specific conditions assayed in the study; the developed UAE procedure could be suitable and adequate to obtain the dried extract product enriched in these bioactive compounds – OA and UA with a minimal amount of accompanying other natural compounds. The content of UA and OA in apple pomace was not significant different from those obtained in the report[21,45] and higher by the method described in this work.[58]

CONCLUSIONS

Hence, the developed two-stage ultrasound-assisted extraction procedure combined with quantitative determination HPLC method of triterpenoid acids – OA and UA is simple, effective, eco-friendly, reproducible and high-yield technique, which provide high quality of target compounds in the dried powdered product form. The extraction efficiency for isolation of these triterpene acids from the apple processing agro-industrial waste materials depends on the nature and the volume of the selected extraction solvent, solubility of target products in the solvent, the extraction time and the sample size. The research results can be used to design a technological process and establish the optimal parameters to obtain standardized form of target products from specific raw materials in terms of the triterpene acid-based medicinal content. Also, the developed and validated HPLC method for quantitative determination of oleanolic acid and ursolic acid is rapid, effective, sensitive and specific analytical procedure which can be successfully used by scientific and quality control laboratories.

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