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Ultrasound-Assisted Extraction- and Liquid Chromatography-Based Method Development and Validation for Obtaining and Qualitative Determination of Apple Pomace Three Triterpene Acids using Analytical Quality by Design

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ABSTRACT

Background: Apple pomace has garnered significant attention within the life sciences domain due to its underutilized status as a waste material from apple processing. It represents a cost-effective and abundant source of triterpene acids due to its multifunctional clinical, nutritional, and pharmaceutical benefits. **Purpose:** The present study aimed to develop and validate a new, selective, effective, robust and reproducible laboratory methodology based on extraction, purification and analytical procedures to obtain and determining three major triterpene acids – Ursolic acid (UA), Oleanolic acid (OA) and Betulinic acid (BA) into the dry extracted product from apple pomace. **Method:** A new, cost-efficient, rapid, selective and high-yield two-stage ultrasound-assisted extraction procedure was developed and the effect of critical parameters: ultrasonic power, extraction time, solvent volume, temperature, and the amount of raw material on the extraction process were investigated. The dry column vacuum chromatography technique was used for purification to remove unwanted non-polar and polar impurities from the target bioactive compounds; A new, effective, specific, sensitive, and rapid HPLC analytical procedure was developed using analytical quality by design (AQbD) approach and validated according to ICH guidelines. **Conclusion:** The method has a good accuracy (the mean recovery >95 %) and linearity (R2>0.999). The limit of quantitation (LOQ) is 0.0001 mg/mL for UA, 0.00005 mg/mL for OA and 0.000025 mg/mL for BA. The validation results confirm that the method is specific, precise and robust. The purity of the extracted and purified target product from apple pomace is not less than 93 %. The developed laboratory methodology is capable of being considered for industrial purposes and through the appropriate technology transfer process can be successfully transferred to the industrial scale.

Keywords: Ultrasound-assisted extraction, HPLC, Validation, Triterpene acids.

Introduction

In the contemporary context, global apple production is on an upward trajectory, with consumption rates maintaining a relatively stable pattern [1]. Approximately 70–75% of apples are consumed fresh, while the remainder, constituting 25–30% of the global apple yield, is processed into a variety of value-added products such as juice, wine, jam, and dried goods [2]. Among these, apple juice emerges as the preeminent apple-derived product, comprising 65% of all processed apple products. It is estimated that about 75% of the apple's fresh weight is converted into juice during the production process, leaving behind a substantial amount of by-product, colloquially termed pomace [1]. This by-product predominantly consists of apple peels, seeds, and stems, with an approximate compositional breakdown of 95%, 2-4%, and 1%, respectively [3]. Recently, apple pomace has garnered significant attention within the life sciences domain due to its underutilized status as a waste material from apple processing [4]. It represents a cost-effective and abundant

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source of fruit-derived bioactive compounds, boasting potential for exploitation due to its multifunctional clinical, nutritional, and pharmaceutical benefits. The conversion of apple processing waste into high-value products not only holds economic and health significance but also offers a sustainable solution to mitigate environmental concerns [4]. Notably, apple pomace is rich in pentacyclic triterpene acids, such as Ursolic acid (UA), Oleanolic acid (OA), and Betulinic acid (BA), which have been the focus of extensive research [1, 5]. These compounds are celebrated for their minimal toxicity and potent pharmacological properties, encompassing a wide spectrum of activities including anticancer, chemopreventive, hepatoprotective, antiviral, antibacterial, anti-inflammatory, anticardiovascular, anti-atherosclerotic, antidiabetic, antioxidant, immunomodulatory, neuroprotective and gastroprotective effects [1, 2, 4-9]. Additionally, OA and UA find applications in the manufacturing of food and sports supplements, as well as critical components in cosmetic formulations, underscoring the versatile and beneficial nature of apple pomace-derived compounds [10-11]. The chemical structures of these compounds are given in Figure 1.

The number of articles per year relative to apple pomace utilization for obtaining triterpene acids has increased greatly in the last decade. Currently, the extraction methods of

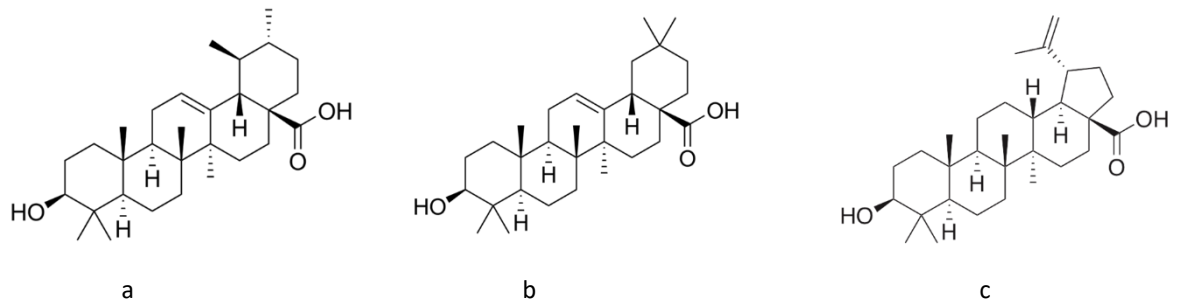

Figure 1 | The chemical structures of ursolic (a), oleanolic (b) and betulinic (c) acids.

triterpene acids include different techniques such as Soxhlet extraction [12], heat reflux extraction, microwave-assisted extraction, accelerated solvent extraction [13] and supercritical fluid extraction [14]. Various methods of increasing interest for determining and obtaining triterpene acids from apple reprocessing materials and other plant resources can be found and reviewed. The authors have been examined the application of high-speed counter-current chromatography to ursolic acid-rich substrates, represented by the pre-purified peel extracts of four varieties of apples using chloroform, ethyl acetate and ethanol [15]. A simple method has been proposed by the authors to obtain UA by crystallization and recrystallization of the ethanol extract of Clinopodium revolutum [16]. The researchers carried out multi-stage extraction of UA with ethanol, chloroform, hexane from apple peels and purification and analysis using column chromatography combined with ultraviolet-visible spectroscopy and high-performance thin-layer chromatography [17]. The study concerns the evaluation of triterpene profiles, the quantitative composition of different parts of apple fruit and High-Performance Liquid Chromatography (HPLC) analyses of triterpenes in apple sample matrices [18]. The 15 edible hydrophobic deep eutectic solvents were used to extract UA from apple peel in the paper proposed by the authors [19]. Other researchers suggest the evaluation of triterpene profiles and the quantitative composition of different parts of apple fruit from 17 various origin and vigor rootstocks using HPLC [20]. The authors have been proposed an extraction method with 2% NaOH/ethanol followed by filtration and acidification for obtaining triterpene acid from dried apple peels [21]. Compared to other methods, ultrasonic extraction, as an emerging extraction method, is widely favored by researchers due to its advantages of high extraction efficiency, low cost, and low energy consumption [3, 22-27]. Its use of ultrasonic vibration can dissolve the required extract. Based on the acoustic principle, cavitation force as the main driving force can produce continuous compression under the action of a solvent. The formation of internal pressure microbubbles causes a “micro-explosion”. These produce small but significant shockwaves that produce subsequent releases of bioactive compounds from plant material [22].

The establishment of optimal parameters for the extraction process, alongside a thorough investigation into the various factors influencing this process and the maximization of target compound recovery, necessitates the deployment of an analytical methodology characterized by appropriateness, precision, specificity, sensitivity, and accuracy. Such a methodology is essential for the accurate quantification of analytes in both the extracted product and the raw material. In the context of triterpene acid analysis, HPLC technique has gained recognition as a preferred analytical technique for the quantitative assessment of bioactive compounds within sample matrices. A review of the existing literature indicates a notable lack of studies detailing the use of the Ultrasound-Assisted Extraction (UAE) method in conjunction with chromatographic techniques for the effective and quantitative extraction,

purification and determination of triterpene acids from apple-processing agro-industrial waste. This gap highlights the need for methodological advancements to enhance the analytical utility of these processes for such purposes. In addition to the above, there is also insufficient information in the literature in terms of the practical application of existing methods, their ability to be successfully transferred from the laboratory scale to the industrial one. Therefore, it is very important in the context of method development. The method development process should take into account the feasibility of use for industrial purposes as much as possible. The objective of this research was to develop a new, cost-efficient, rapid, selective, reproducible, and high-yield extraction methodology through the innovative application of a two-stage UAE technique. This technique aimed at the efficient isolation of three major triterpene acids – UA, OA, and BA – from apple pomace. Additionally, the study sought to establish a new, effective, specific, sensitive, and rapid HPLC analytical procedure for the quantitative determination of these compounds within both the extracted product and the residual waste materials. This paper also presents a validation study of the developed method, ensuring its reliability, robustness, and sustainability. To achieve these objectives, an Analytical Quality by Design (AQbD) approach was employed, underpinning the method development process with a framework designed to guarantee analytical excellence and adaptability according to a new ICH guideline [28].

Materials and methods

Materials and Reagents

Apple pomace as an apple processing waste material was provided by local manufacturer, Shida Kartli region, Georgia. The waste material was crushed using a chopper and dried in laboratory room protected from direct sun light under the established environmental conditions (the temperature was 19-25ºC and the relative humidity – <60%) during 14 days and then dried at 40ºC in a thermostat for 6-8 hours. The dried samples were ground using a laboratory mill to be powdered and stored in the bottles with closure before extraction. The certified analytical standard of ursolic, oleanolic and betulinic acids, the analytical grade ethyl acetate, hexane, acetone, ethanol, methanol, 2-prepanol, acetonitrile, hydrochloric acid, sodium hydroxide, Merck silica Gel 60 – 0.015-0.040 mm - №1.15111.1000 and Celite® 545 particle size 0.02-0.1 mm were purchased from Merk (Germany).

Equipment and Methods

The chromatographic analysis was performed using LC-20AD Prominence Shimadzu HPLC System (Japan) and the column - Agilent SB-C18 4.6×250 mm, 5 µm (USA). The Milli Q Adventage A10 purification system (Millipore, France), Dual-frequency ultrasonic bath DW-5200DTS (China), Elmasonic P 60H (Germany), Vortex-Genie™ 2 Mixer (USA), pH-meter Hanna Instruments HI 2211 (USA), BIOBASE Small Capacity Rotary Evaporator (China), GFL water bath (Germany), Hermle Z200A Centrifuge (Germany), Analytical balance ALX-210 (USA), laboratory mill SM-450C were used for sample preparation.

The ultrasound frequencies were 25 and 37 kHz; the temperature was controlled at 25-60ºC during ultrasonication. Ethyl acetate, ethanol, acetone and 2-propanol were selected as non-toxic and the best extraction solvents for triterpene acids. The two-stage UAE was carried out according to the following procedure: 5-50 g of the powdered dried sample was transferred to a 500 mL volume conic flask; 300 mL of acidic purified water (pH 2 with 1 M hydrochloric acid) was added and ultrasonicated for 30 min at 37 kHz and 50^{0}C (UAE stage I). The crude extract suspension was centrifuged at 4000 rpm for 10 min. The wet sample was washed twice with 300 mL of purified water at 60^{0}C. The obtained aqueous suspension was centrifuged at 4000 rpm for 10 min. The obtained residue was separated from the supernatant; then 100-300 mL of ethyl acetate/a mixture of ethyl acetate and acetone/2-prapanol 80:20 v/v was added to the obtained residue after centrifugation, mixed vigorously for a few minutes and ultrasonicated for 20-40 minutes at 25/37 kHz and 25-40^{0}C (UAE stage II). The crude extract suspension was centrifuged at 4000 rpm for 10 min and the obtained residue was separated from the supernatant. The supernatant was evaporated on a rotary evaporator at 60^{0}C and the dried solid sample was dissolved in 100 mL of hot alkaline ethanol - a mixture ethanol and strong 1 M sodium hydroxide solution 90:10 v/v (pH 10.00±0.05). Then the pH value of this solution was adjusted to 7.00±0.05 with 1 M hydrochloride acid solution (10 mL) and the obtained solution was allowed to stand for 24 hours. The obtained crystalline precipitate was separated from the solution through centrifugation, and then the precipitate was dissolved in 20 mL of ethyl acetate and 3 g of celite was added to the solution. Then solvent was evaporated on a rotary evaporator at 600 °C and the dried solid sample was used for the next clean-up stage. The purification procedure was performed using the dry column vacuum chromatography (DCVC) which is composed of cylindrical sintered glass funnel (height - 10 cm, diameter – 4 cm), a separating funnel, a glass joint connecting these two with a sidearm to apply a vacuum and aspirator pump. Merck Silica Gel 60 – 0.015-0.040 mm - Merck № 1.15111.1000 was used as an adsorbent (height - 3.5 cm for the adsorbent; 1 cm for the eluent). Ethyl acetate and n-hexane were used as eluents with a volume of 20 mL; Gradient elution was from 0% to 55%; the target products were eluted at 25-45 % fractions.

The chromatographic analysis was performed using the LC-20AD Prominence Shimadzu HPLC System (Japan) and a column - Agilent SB-C18 4.6×250 mm, 5 µm (USA) with an isocratic elution of mobile phase (MP) containing a mixture of acetonitrile (ACN) and methanol (MeOH) 80:20 v/v, filtered through PVDF 0.45 µm membrane filter and degassed; The flow rate of MP was 0.5 mL/min; The UV-spectrophotometric detection was performed at 205 nm; The injected volume was 20 µL; The column temperature was maintained at 25°C.

Standards and Sample Preparation

Analytical standards of UA, OA, and BA, diluted in methanol, served as the standard solutions at a concentration of 0.1 mg/mL. This concentration was also employed for the system suitability test solution, utilizing a mixed standard solution. The test solution comprised the dried extracted product, similarly diluted in methanol to maintain uniform concentration. Subsequent filtration of this solution was performed using a 0.45 µm polyvinylidene difluoride (PVDF) microporous membrane filter to ensure purity. Furthermore, to evaluate the method's accuracy, a spiked test solution was meticulously prepared by combining the analytical standards of UA, OA, and BA with an apple pomace sample to achieve analyte concentrations of 50% (6 g of apple pomace sample+26 mg of UA+15 mg of OA+2 mg of UA; concentrations approximately: 0.0225 mg/mL, 0.0125 mg/mL, 0.00175 mg/mL, respectively), 100% (10 g of apple pomace sample+43 mg of UA+25 mg of OA+4 mg of UA; concentrations approximately: 0.045 mg/mL, 0.025 mg/mL, 0.0035 mg/mL, respectively), and 150% (15 g of apple pomace sample+65 mg of UA+38 mg of OA+5 mg of UA; concentrations approximately: 0.075 mg/mL, 0.0375 mg/mL, 0.00525 mg/mL, respectively), within the spiked solution. Quantification was executed via the external standard method. For background control, a blank solution of the diluent, methanol, was used, ensuring a comprehensive assessment of the method's precision and reliability.

Calculations

The concentration of each analyte – C_S, mg/mL (the extraction yield) in the test solution/the spiked test solution was calculated by the following formula:

$$C_s = \frac{A_s \times W_{st} \times P}{A_{st} \times V_{St} \times 100} \quad (1)$$

where, A_S – The peak area of UA/OA/BA obtained with the test solution/the spiked test solution; A_{st} – The peak area of UA/OA/BA obtained with the standard solution; WSt – The weight of the standard, mg; V_{St} – The dilution of the standard, mL; P – The purity of the standard on anhydrous basis, % (Standard's potency from the certificate of analysis).

The percentage of UA/OA/BA (the purity) – X_P, % in the extracted product was calculated by the formula:

$$X_P = \frac{C_s \times V_S \times 100}{W_s} \quad (2)$$

where, W_S - the weight of the extracted product sample, mg; V_S – the dilution of the extracted product sample, mL.

The content of UA/OA/BA – X_i (the extraction yield), mg per 1 g of the dried sample of apple pomace was calculated by the formula:

$$X_i = \frac{A_s \times W_{st} \times V_2 \times W_1 \times P}{A_{st} \times W_s \times W \times V_{St} \times 100} \quad (3)$$

where, W - the weight of the dried sample of apple pomace, g;

The percentage recovery - R, % was calculated by the following formula:

$$R, \% = \frac{W_d \times 100}{W_a} \quad (4)$$

where, Wd - the determined amount of UA/OA/BA, mg, which was calculated $W_d=W_{sp}-W_0$; W_0 - the endogenous amount of each analyte in apple pomace, mg; Wa - the added amount of UA/OA/BA standard, mg.

The similarity factor – Sf, % for two standard solutions was calculated by the following formula:

$$Sf = \frac{W_{st1} \times A_{st2} \times 100}{W_{st2} \times A_{st1}} \quad (5)$$

where, A_{St1} – the peak area of UA/OA/BA obtained with the standard solution I; W_{St1} – the weight of the standard for the standard solution II, mg; W_{St2} – the peak area of UA/OA/BA obtained with the standard solution I; W_{St2} – the weight of the standard for the standard solution II, mg.

Method Validation and Analytical Quality by Design Methodology

The developed analytical HPLC procedure was validated with respect to the following validation parameters: system suitability test (SST), specificity, linearity-range, precision, accuracy and sensitivity according to ICH guidelines and the appropriate methodologies reported by the authors [29-32]. The method development and the robustness test were performed by implementing AQbD principles. The prime goal was to define the analytical target profile (ATP) which means to develop a method based on a combination of a selective, robust, high-yield UAE and robust, accurate, specific, sensitive, reproducible HPLC procedures. The ATP was fixed based on acceptable criteria of ICH guidelines to achieve the goal of this study [28]. The design of experiments (DoE) was applied to identify the significant effect of variables such as critical method parameters (CMPs) and critical process parameters (CPPs) on critical analytical attributes (CAAs) and critical quality attributes (CQA) of the extracted product. The established acceptance criteria and requirements of all the validation parameters were defined as the CAAs and the physical-chemical properties, such as a high-quality and purity of the dried extracted product (the purity ≥90%; total residual solvent <5000 ppm) were defined as the CQAs [28, 31, 33-34].

All the process and method parameters were assessed and selected using a risk assessment approach. Each parameter was considered as a factor or variable affected on the CAAs and the CQAs and evaluated by the following risk parameters: 1) risk severity (S) (direct impact assessment; risk level: major, moderate, minor); 2) risk probability (P) (how often and actually it is possible for it to occur; risk level: very unlikely in case of an automatically controlled parameter, occasional – a manually controlled parameter and regular – an experimental parameter); 3) risk detectability (D) (how to detect it in the process; risk level: normally not detected, likely detected,

regularly detected). All the variables that represented the parameters of the method were grouped into critical, significant and negligible categories; accordingly, critical, important and negligible method parameters were identified. The critical independent parameters or variables (CPPs and CAAs) – Ki with two high and low levels ("+" and "-") of the nominal values ("0") (the normal operating condition - NOP) were involved in the DoE and the dependent variables used as responses for the assessment of the robustness test of the method. The determined operating range (from "+" to "-") of each critical parameter represents a proven acceptable range for analytical procedure named the method operable design region (MODR) according the AQbD principles. The MODR combines ATP requirements and the probability that programs meet these criteria with predictive models on the DoE and is validated throughout the procedure lifecycle and refined as needed when new knowledge is gained. The method operable region and continuous improvement process provide robust analytics with regulatory flexibility. The experiments were conducted in N-runs according to the Placket-Burman design ($k<N-1$; where, K – the number of variables and N – the number of experiments) [28, 30-34]. The concentration of UA/ OA/BA (Cs), mg/mL as a yield of extraction and the SST parameters – the column efficiency (theoretical plates – N), the tailing factor (USP symmetry – the coefficient of the peak symmetry S=W0.05/2f), the resolution (Rs) were used as responses or the dependent variables. The variation of each variable and the modality of the obtained data were evaluated.

Results and Discussion

Ultrasound-Assisted Extraction Procedure

To enhance the efficiency of the UAE process for extracting compounds from apple pomace, various parameters were evaluated: ultrasonic power, extraction time, solvent volume, temperature, and the amount of apple pomace used. The results are displayed in Figure 2. Experiments were conducted over different durations (10, 20, 30, and 40 minutes) under specific conditions: ultrasonic power at 37 kHz, 20 g of apple pomace, 200 mL of ethyl acetate as the solvent in the second stage of extraction, and a temperature maintained at 40±2ºC. The findings indicated that the yields of UA, OA, and BA followed similar patterns under these conditions, peaking at 30 minutes. The yield notably increased from 20 to 30 minutes and then diminished, with a significant drop noted at 10 minutes. The optimal extraction period was identified as 30 minutes, within which the highest yields of UA, OA, and BA were achieved.

The experiments demonstrated a notable impact of ultrasound frequency on the extraction yield of compounds from biomass, comparing two frequencies: 25 and 37 kHz. Higher yields were achieved with the 37 kHz setting, indicating that this ultrasound frequency accelerates the dissolution equilibrium between the biomass and extraction solvent. The thermal effects of ultrasound were deemed negligible as the generated heat likely dispersed evenly. Yield differences for UA, OA, and BA at these frequencies were 15%, 38%, and 60%, respectively, highlighting a significant variance based on ultrasound frequency. Solvent volume's effect was also studied, using ranges from 100 to 300 mL. Optimal yields were observed with 200 mL of solvent, beyond which yields decreased. This suggests that while a certain volume is essential for dissolving target compounds effectively and quickly reaching equilibrium, excessive solvent volume diminishes yield, especially for OA. For BA, yields increased from 100 to 200 mL and then plateaued, showing stability in extraction yield across solvent volume variations, unlike UA and OA. This stability could be attributed to BA's inherent properties. Therefore, 200 mL was determined as the optimal solvent volume for maximizing extraction yield.

The study explored the impact of sample size on extraction efficiency, utilizing 5, 20, 35, and 50 g of samples. It was found that the extraction yield of target compounds is significantly influenced by the sample size, with the highest yield achieved at a sample size of 20 g.

Temperature's effect on extraction was also examined at 25, 30, and 40°C. Results indicated that the solubility of triterpene acids, and consequently their extraction yield, increased with temperature. However, this temperature effect was not markedly pronounced. Higher temperatures could potentially cause degradation of both target and accompanying compounds, complicating subsequent purification stages. The findings suggest a balance between sample size, extraction time, and solvent volume for optimal extraction efficiency: smaller sample sizes and larger solvent volumes tend to reduce extraction time, but an excessive solvent volume relative to the sample size can be counterproductive. Based on these results, the optimal parameters for the two-step Ultrasound-Assisted Extraction (UAE) from dried and powdered apple pomace samples were identified: ultrasonic frequency at 37 kHz, sample size of 20 g, extraction time of 30 minutes, temperature at 40° C, solvent volume of 200 mL, with ethyl acetate as the solvent for the second extraction stage.

Analytical Procedure and Validation

The final chromatographic conditions of analytical HPLC procedure were determined by optimizing the system operational parameters: the wavelength for detection, the composition of mobile phase, the flow rate, the nature of stationary phase and the injection volume. The system suitability parameters: theoretical plates, tailing factor and resolution were optimized. The chromatographic system performance was checked for the study of each validation parameter. The SST was performed by using six replicate injections (n=6) of the standard solution of UA/OA/BA. The RSD of peak areas - RSDA, the RSD of retention times – RSDRT, the tailing factor (Tf), the number of theoretical plates (N), and the resolution between closely eluting principal peaks on the mixed standard solution chromatogram were measured. The results are summarized in Table 1.

Table 1 | The results of system suitability test and specificity of the method with acceptance criteria (Ac).

Parameter	Ac	UA	OA	BA
Column efficiency (N)	≥2000	>15766	>13178	>13746
RSD_A, % (n=6)	≤1.0	0.113	0.127	0.105
RSD_{RT},% (n=6)	≤1.0	0.018	0.024	0.013
Tailing factor (S)	0.85-1.5	0.93	0.98	1.02
Peak purity	≥0.990	0.999	0.999	0.999
Match factor	≥995	999	999	999
Resolution (R_s)	≥0.85	0.89	1.82	-
$Diff._{RT}$, %	≤1.0	0.251	0.145	0.312

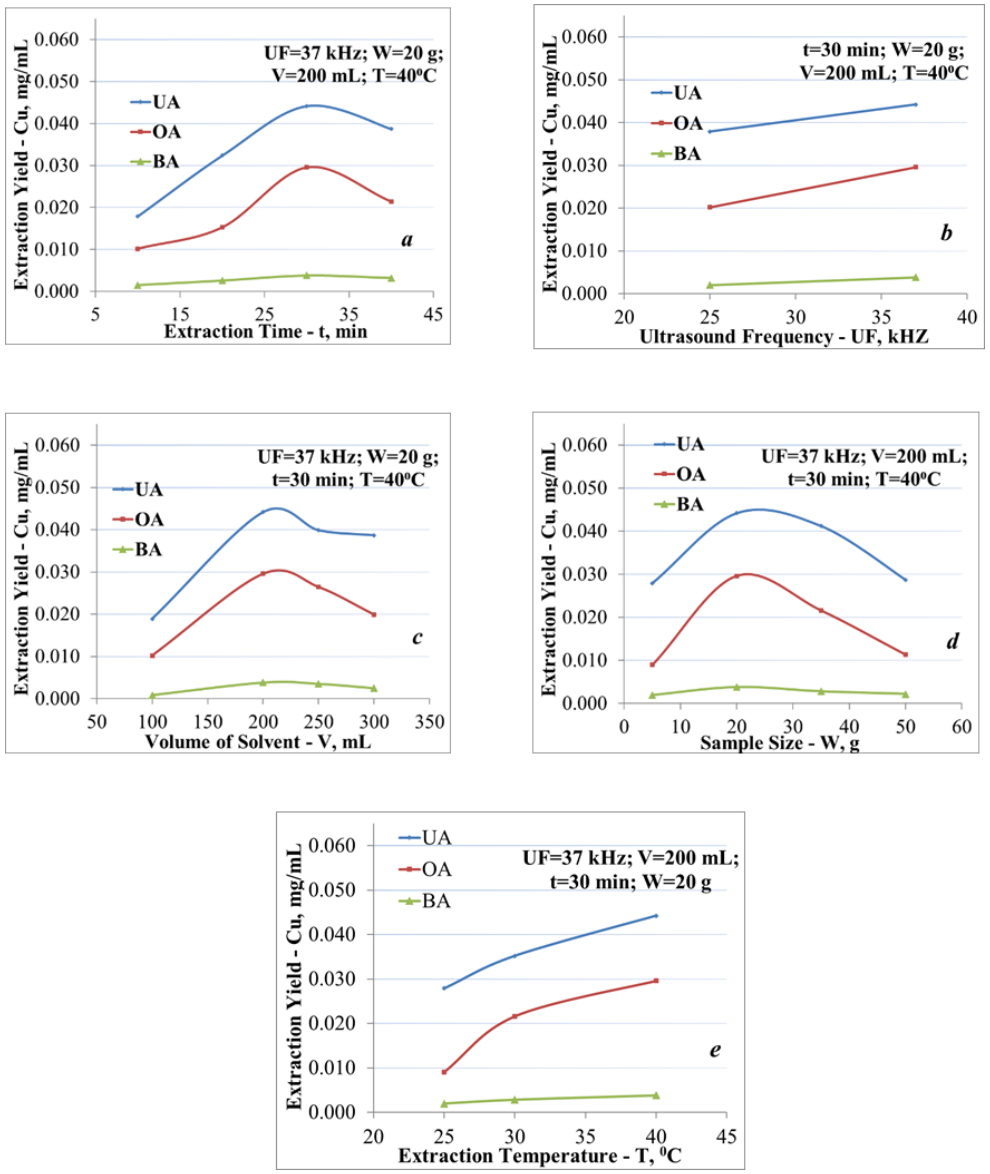

Figure 2 | The effect of different extraction parameters on the extraction yield (Cs) of UA, OA and BA: the effect of the extraction time (t=10-40 min) at UF=37 kHz, W=20 g, V=200 mL, T=40^{0}C (a); the effect of the ultrasonic power (UF=25, 37 kHz) at t=30 min, W=20 g, V=200 mL, T=40^{0}C (b); the effect of the volume of extraction solvent (V=100-300 mL) at UF=37 kHz, t=30 min, W=20 g, T=40^{0}C (c); the effect of the sample size (W=5-50 g) at UF=37 kHz, t=30 min, V=200 mL, T=40^{0}C (d) and the effect

The specificity test was checked by injecting the standard solution, system suitability test solution, test solution and background control - blank (diluent) solution. According to the obtained analytical data there was no interference from the diluent and secondary peaks from the test solution at the retention time (RT) of the analyte peaks; the peaks represented UA, OA, BA only, and there was not observed a co-elution. The peaks of UA, OA and BA were pure and purity factors were greater than purity threshold values. Acceptance criteria (Ac) was ≥995.0. The percentage difference (DiffRT, %) between the retention times and the match factor value between the UV-Vis spectra obtained from the standard and test solutions were evaluated. None of the spectra between peak start and peak end deviated from the spectrum at the peak maximum which confirms a very strong spectral similarity (Ac: ≥0.990). Figure 3 and 4 depicts the chromatogram and overlay UV-Vis absorption spectra at 200-800 nm obtained with the system suitability test solution, respectively. Hence, this analytical procedure has a high specificity.

In order to study the linearity-range, working standard solutions were prepared at different concentration levels (the concentration range was 0.0001-0.5 mg/mL for UA, 0.00005-0.5 mg/mL for OA, 0.000025-0.5 mg/mL for BA by three replicates (n=3). The linearity was checked by the square of correlation coefficient – R2 (Ac: ≥0.998), the RSDA (Ac: ≤2.0%) at all concentration levels excluding the last concentration level which should not be more than 10% and the RSDRT (Ac: ≤1.0%). The calibration curve (linearity graph) was constructed by plotting the average peak areas against the corresponding concentrations of the injected working standard solutions which is given in Figure 5. The sensitivity of the analytical procedure was determined with respect to the limit of quantitation (LOQ) and the limit of detection (LOD).

The s/N ratio should be ≥10 for the LOQ, ≥3 for the LOD. The LOQ was achieved by injecting a series of stepwise diluted solutions and the precision was established at the specific determined level. The RSDA should not be more than 10.0% (Ac). The determined LOD and LOQ of the procedure are presented in Table 2. Hence, the analytical procedure is a linear and sensitive.

The precision parameter was estimated by measuring repeatability (intra-day precision) and time-dependent intermediate precision (inter-day precision) on six replicate injections of the standard solution and on six individual determinations of UA/OA/BA in the test solutions at 100 % concentration. The system precision was checked by the RSD_A (Ac: ≤1.0 %) of retentions times and the RSD_{RT} (Ac: ≤1.0 %) of peak areas obtained with the standard solution and the method precision by the RSD of determined concentrations (mg/mL) of each analyte in the test solution (Ac: ≤10.0 %). The intermediate precision was carried out on a different day using the same type column with a different serial number and the same samples of the extracted product. The precision was checked by the cumulative RSD, % of twelve individual determinations (total inter-day and intra-day determinations) of analytes (Ac: ≤10 %), the percentage difference (Diff, %) between inter-day and intra-day average results (Ac: ≤10 %), the value of the F-test (Ac: F_{crit}≤5.05) and the value of the t-test

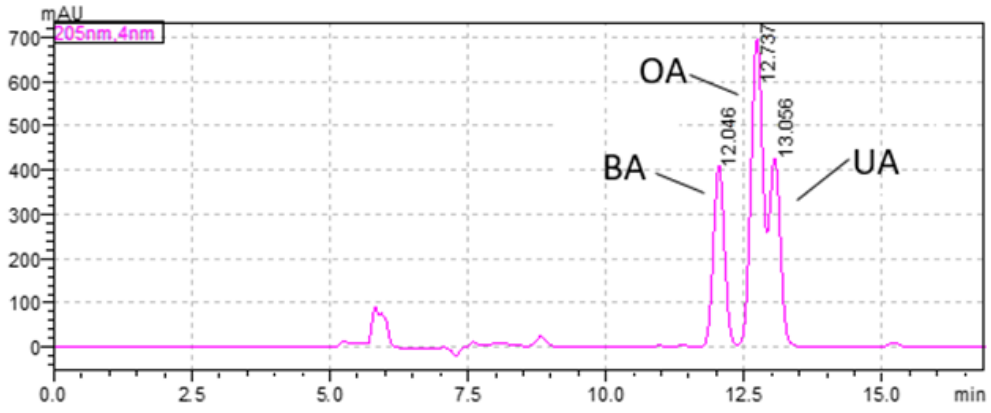

Figure 3 | The chromatogram detected at 205 nm and obtained with the system suitability test solution (RT=12.046 min corresponds to BA; RT=12.737 min - OA; RT=13.056 min - UA).

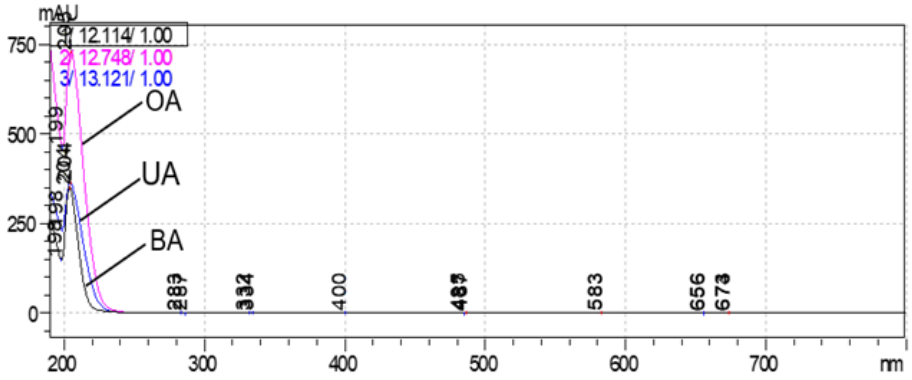

Figure 4 | The overlay UV-Vis absorption spectra scanned in a wavelenght range of 190-800 nm and obtained with the system suitability test solution.

(Ac: $t_{crit} \leq 2.23$). The results obtained with the system and method precision studies are given in Tables 3, 4. Hence, the procedure has a good precision.

The accuracy test was assessed by performing recovery studies using the standard addition method by spiking the known amounts at 50%, 100 %, 150% of standard with three replicate injections (n=3). The accuracy was expressed as a percentage recovery which was the percentage of standard compound recovered from the spiked test solution (sample + standard) with a corresponding RSD, %. The percentage recovery - Rn, % was determined by injecting two standard solutions, the test solution, three spiked test solution with three replicate injections (n=3). The mean recovery of the method – R, % including extraction procedure should be within 95.0 –105.0% (Ac), also the RSD of the percentage recoveries (n=3×3=9) should be <5.0% (Ac). The similarity factor (Sf) between two standard solutions was calculated and should be within 98.0 %-102.0 % (Ac). The results of the recovery are given in Table 5.

The robustness test was carried out by the small changes in the determined critical parameters (Ki) - CPPs and CAAs as the independent variables with the method operable regions

Table 2 | The results of method sensitivity, limits of quantitation and detection with acceptance criteria.

Parameter	Ac	Value		
		UA	OA	BA
LOQ, mg /mL		0.00010	0.000050	0.000025
LOD, mg /mL		0.00005	0.000025	0.000010
RSD_A, % for LOQ (n=6)	<10.0 %	8.001	5.343	4.65
RSD_{RT} ,% for LOQ (n=6)	<1.0 %	0.050	0.073	0.012
s/N for LOQ	≥10	11.23	13.15	12.25
s/N for LOD	≥3	6.05	7.98	5.01

Table 3 | The results of the system precision (repeatability and intermediate precision).

Analyte	Repeatability (intra-day)		Intermediate precision (inter-day)	
	RSD_A (n=6)	RSD_{RT} (n=6)	RSD_A (n=6)	RSD_{RT} (n=6)
UA	0.825	0.144	0.150	0.895
OA	0.745	0.321	0.166	0.931
BA	0.433	0.277	0.391	0.744

Table 4 | The results of the method precision (repeatability and intermediate precision).

Test solution №	Concentration, mg/mL					
	Repeatability (intraday)			Intermediate Precision (Inter day)		
	UA	OA	BA	UA	OA	BA
1	0.0442	0.0296	0.0038	0.0429	0.0242	0.0035
2	0.0483	0.0268	0.0035	0.0450	0.0265	0.0035
3	0.0449	0.0267	0.0034	0.0438	0.0244	0.0038
4	0.0427	0.0278	0.0036	0.0438	0.0261	0.0039
5	0.0433	0.0287	0.0036	0.0447	0.0257	0.0037
6	0.0452	0.0263	0.0038	0.0477	0.0245	0.0036
Average	**0.0224**	**0.0138**	**0.0018**	**0.0223**	**0.0126**	**0.0018**
RSD, %	**8.845**	**9.457**	**8.525**	**7.448**	**7.878**	**9.871**
Average (n=12)				**0.0447**	**0.0264**	**0.0036**
RSD, % (n=12)				**3.900**	**6.367**	**4.448**
F-test (0.05;5;5)				**1.41**	**1.73**	**1.37**
t-test (0.05;10)				**0.8**	**2.17**	**0.42**
Diff, %				**0.21**	**9.18**	**1.24**

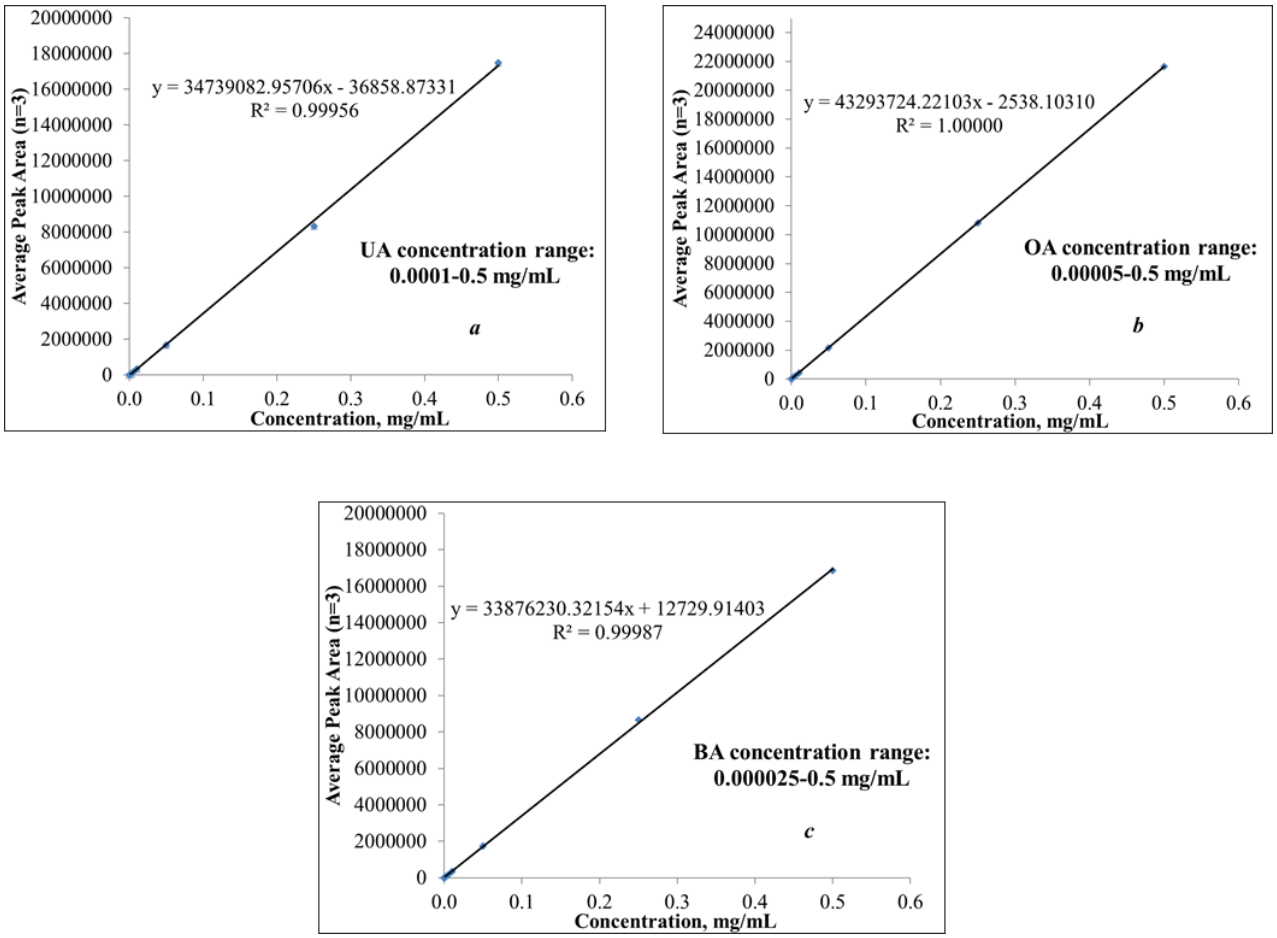

Figure 5 | Calibration curves (linearity graphs) of UA in the concentration range of 0.0001-0.5 mg/mL (a), OA in the concentration range of 0.00005-0.5 mg/mL (b) and BA in the concentration range of 0.000025-0.5 mg/mL (c) with linear equation and the square of correlation coeficient.

Table 5 | The results of the accuracy test checked at three (50, 100, 150 % of nominal concentration) concentration levels off UA, OA, BA with acceptance criteria.

Analyte	Sf, %	Average Recovery (R_n), %			R, % (n=9)
		50 % (n=3)	100 % (n=3)	150 % (n=3)	
OA	99.25	96.05	96.47	95.32	**96.26**
UA	100.10	98.18	97.09	96.17	**97.64**
BA	100.02	97.63	98.45	97.75	**98.04**
Ac	**98.0-102.0**	**95.0-105.0**			

affected on the dependent or response variables of the method. The critical parameters with the method operable regions are summarized in Table 6.

The robustness test was performed by 12-run experiments with 11 factors according to the DoE matrix (Table 7). The variability of the SST parameters was acceptable and none of the mentioned parameters was not out of the acceptance criteria. The variability of the concentrations of UA/OA/BA during experiments was assessed by the percentage difference – Diff, % between the precision (n=12) and robustness (N=12) average results of the determined concentration of UA, OA and BA, mg/mL (extraction yields) which was not more than the acceptance criteria of the precision parameter (Ac: ≤10.0 %). The values of RSD of determined concentrations (mg/mL) of each analyte (N=12) were acceptable (Ac: ≤10.0 %); the values of the F-test (Ac: Fcrit≤4.04) and t-test (Ac: tcrit≤2.12) were evaluated.

The robustness test was performed by 12-run experiments with 11 factors according to the DoE matrix (Table 7). The variability of the SST parameters was acceptable and none of the mentioned parameters was not out of the acceptance criteria. The variability of the concentrations of UA/OA/BA

Table 6 | The critical parameters with the method operable regions.

№	Critical Parameter (CPPs & CAA) - Ki	Unit	Levels of MODR		
			Low level (-)	Nominal level (NOP) (0)	High level (+)
1	Sample size – K1	g	18	20	22
2	Volume of solvent (UAE stage II) – K2	mL	200	250	300
3	Solvents and their ratio (UAE stage II) – K3	% v/v	2-Propanol /Ethyl acetate 20:80	Ethyl acetate 100	Acetone/Ethyl acetate 20:80
4	Extraction time (UAE stage II) – K4	min	25	30	35
5	Solvent (alkali) (clean-up stage) – K5	-	Ethanol/1M NaOH 92:8	Ethanol/1M NaOH 90:10	Ethanol/1M NaOH 88:12
6	Volume of solvent (alkali) (clean-up stage) – K6	mL	90	100	110
7	pH of solvent (acid) (clean-up stage) pH – K7	-	6.9	7.0	7.1
8	pH of solvent (alkali) (clean-up stage) pH – K8	-	9.9	10.0	10.1
9	Solvent's ratio in MP – K9	% v/v	ACN:MeOH 75:25	ACN:MeOH 80:20	ACN:MeOH 85:15
10	Flow rate of MP – K10	mL/min	0.4	0.5	0.6
11	DAD detection wavelength – K11	nm	203	205	207

during experiments was assessed by the percentage difference – Diff, % between the precision (n=12) and robustness (N=12) average results of the determined concentration of UA, OA and BA, mg/mL (extraction yields) which was not more than the acceptance criteria of the precision parameter (Ac: ≤10.0 %). The values of RSD of determined concentrations (mg/mL) of each analyte (N=12) were acceptable (Ac: ≤10.0 %); the values of the F-test (Ac: F_{crit}≤4.04) and t-test (Ac: t_{crit}≤2.12) were evaluated.

The results of the robustness parameter show that none of the examined factors in the method operable regions did not have any significant effect on the concentration of each analyte in the test solution. On base of the analytical data obtained the precision and robustness parameters a histogram was plotted to indicate the distribution of variable values (N=24) (Figure 6). There is a multi-modal data distribution which shows that analytical data are collected from more than one procedure or condition. The results experimentally confirm the existence of several critical factors and the complexity of the method, although the established variability does not go beyond acceptable limits and proves the robustness. The analytical data spread varies within acceptable criteria.

Within the robustness test, the standard solution stability and membrane filter compatibility were studied. The stability 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 Diff, % between the peak areas of the standard solution stored and freshly prepared one. The standard solution was stable within 7 day – Diff, %=2.35 % for UA; 2.51 % for OA; 1.65 % for BA (Ac: ≤3 %). The compatibility of the used membrane filter - PVDF was evaluated using the standard solution of each analyte and by calculating the Diff, % between peak areas of filtered and non-filtered standard solutions. The calculated Diff, % was 0.71 % for UA, 0.56 % OA, 0.74 % for BA (Ac: ≤2 %) which gives confidence that the adsorption of each analyte does not occur on the used filter and affect on the result of the analysis.

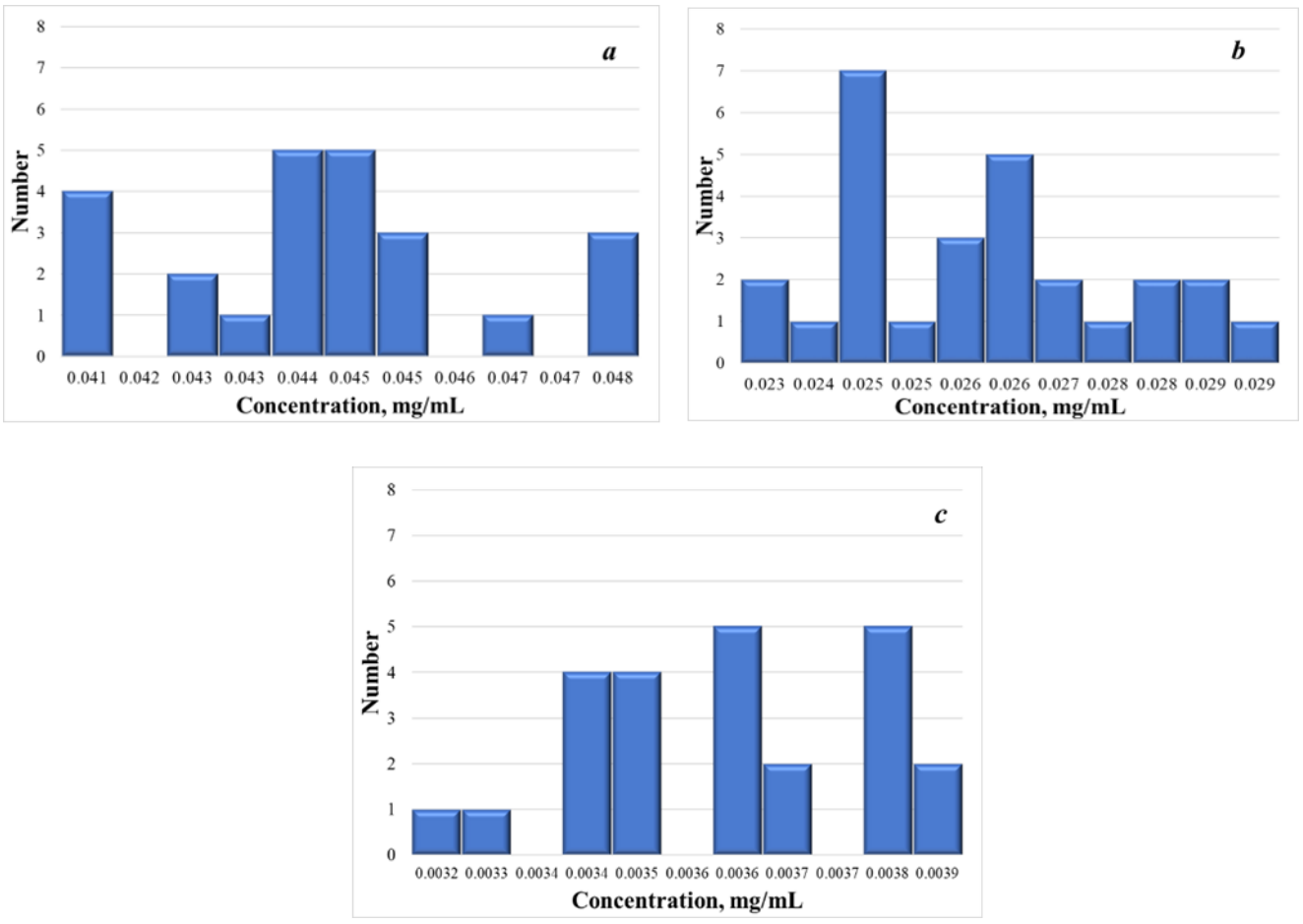

Figure 6 | Histograms of the determined concentrations (extraction yields) of UA (a), OA (b) and BA (c) in the test solutions obtained under normal and changed conditions (n=24).

Table 7 | The results of 12-run experiments for the robustness parameter.

Run (N) №	Dependent Variable - Ki											Analyte	Response Variables	
	K1	K2	K3	K4	K5	K6	K7	K8	K9	K10	K11		Cs, mg/mL	SST
1	+	-	+	+	+	-	-	-	+	-	-	UA	0.0412	**UA:** N>14205 S=0.92-0.98; **OA:** N=14774 S=0.96-1.01 **BA:** N=15774 S=0.95-1.01; **BA/OA:** Rs>0.88 **OA/UA:** Rs>1.81
												OA	0.0256	
												BA	0.0037	
2	+	-	+	+	+	-	-	-	+	-	+	UA	0.0445	
												OA	0.0253	
												BA	0.0034	
3	-	+	+	+	-	-	-	+	-	+	+	UA	0.0454	
												OA	0.0284	
												BA	0.0036	
4	+	+	+	-	-	-	+	-	+	+	-	UA	0.0412	
												OA	0.0233	
												BA	0.0038	
5	+	+	-	-	-	+	-	+	+	-	+	UA	0.0413	
												OA	0.0245	
												BA	0.0036	
6	+	-	-	-	+	-	+	+	-	+	+	UA	0.0411	
												OA	0.0230	
												BA	0.0038	
7	-	-	-	+	-	+	+	-	+	+	+	UA	0.0477	
												OA	0.0255	
												BA	0.0035	
8	-	-	+	-	+	+	-	+	+	+	-	UA	0.0445	
												OA	0.0262	
												BA	0.0034	
9	-	+	-	+	+	-	+	+	+	-	-	UA	0.0438	
												OA	0.0244	
												BA	0.0033	
10	+	-	+	+	-	+	+	+	-	-	-	UA	0.0438	
												OA	0.0261	
												BA	0.0034	
11	-	+	+	-	+	+	+	-	-	-	+	UA	0.0452	
												OA	0.0244	
												BA	0.0039	
12	-	-	-	-	-	-	-	-	-	-	-	UA	0.0465	
												OA	0.0245	
												BA	0.0032	
Analyte													**UA**	**OA**	**BA**
Average concentration, mg/mL													0.0439	0.0251	0.0035
RSD, % (n=12)													5.100	5.748	6.183
Diff, %													1.93	5.22	2.40
F-test (0.05;5;11)													0.78	0.80	0.53
t-test (0.05;16)													0.77	2.09	0.60

Table 8 | The content of triterpene acids in apple pomace and the extracted product.

Analyte	The content of triterpene acid in the apple pomace - X_i, mg/g	The content in the extracted product - X_p, % (m/m)
UA	4.299	59.78
OA	2.543	35.36
BA	0.349	4.86
Total triterpene acids	7.191	93.48

Results of Analysis

The percentage content of UA, OA and BA (the purity) in the extracted dried product obtained with the developed two-stage UAE procedure was estimated. The content of the target compound expressed in mg per 1 g of the dried sample of the raw material (apple pomace) was calculated. The results are given in Table 8.

Concluding Remarks

The developed simple, effective, cost-effective, reproducible and high-yield, two-stage ultrasound-assisted extraction-based method combined with a new rapid, effective, sensitive and specific quantitative determination HPLC procedure for obtaining and controlling the purity of triterpene acids – ursolic, oleanolic and betulinic acids is an alternative technique that provides a high-quality target compound in dried powdered product form. The extracted dried product contains the target compounds with high purity (>93 %). The extraction efficiency depends on the nature and volume of the selected extraction solvent, the solubility of the target product in the solvent, the extraction time and the sample size. The high purity of the extracted product and the validated HPLC procedure give the opportunity to separate and obtain pure triterpene acids from the extracted product using a fractional collector equipment for preparative purposes. Also, this analytical procedure could be successfully applied by scientific and quality control laboratories to determine triterpene acids in apple processing waste materials and the extracted products. The principles and design of analytical method validation studies using AQbD approach could be applied by researchers and specialists working in the field of analytical studies of food and drug products. The developed laboratory methodology is capable of being considered for industrial purposes and through the appropriate technology transfer process can be successfully transferred to the industrial scale.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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