

## AVALIAÇÃO DA INCERTEZA DE MEDIÇÃO DE MÉTODO ANALÍTICO PARA DETERMINAÇÃO QUANTITATIVA DE ÁCIDOS URSÓLICO E OLEANÓLICO NO PROCESSAMENTO DE MAÇÃS DE RESÍDUOS AGROINDUSTRIAIS

## MEASUREMENT UNCERTAINTY EVALUATION OF ANALYTICAL METHOD FOR QUANTITATIVE DETERMINATION OF URSOLIC AND OLEANOLIC ACIDS IN APPLE PROCESSING AGROINDUSTRIAL WASTE MATERIAL

ვამლის გადამუშავების აგროინდუსტრიულ ნარჩენ მასალაში ურსოლისა და ოლეანოლის მჟავების რაოდენობრივი განსაზღვრის ანალიზის მეთოდის გაზომვის განუსაზღვრელობის შეფასება

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## RESUMO

**Introdução:** O bagaço de maçã representa uma fonte de barata e rica em compostos bioativos com propriedades valiosas - ácido ursólico (UA) e ácido oleanólico (OA). Devido à ampla gama de aplicações nas indústrias farmacêutica e nutracêutica, esses compostos possuem alto valor comercial, e possuir um método analítico adequado com incerteza de medição é de grande importância e praticidade. **Objetivo:** O objetivo do presente trabalho foi estimar detalhadamente a incerteza de medição para o método de HPLC validado combinado com o procedimento de extração para a determinação de ácido ursólico e ácido oleanólico do bagaço da maçã. **Métodos:** A análise cromatográfica foi realizada utilizando o sistema LC-20AD Prominence Shimadzu e a extração assistida por ultrassom foi realizada utilizando o banho ultrassônico DW-5200DTS para obter e determinar a concentração de ácido ursólico e ácido oleanólico em bagaço de maçã. O processo de avaliação da incerteza de medição foi realizado pelo diagrama de Ishikawa e uma combinação das abordagens *bottom-up* e *top-down*. **Resultados:** O teor de ácido ursólico e ácido oleanólico (mg/g) em bagaço de maçã com o valor da incerteza expandida foi calculado, determinado em  $7,06 \pm 0,647$  mg/g ( $k=1,96$ ;  $P=95\%$ ) e  $4,70 \pm 0,422$  mg/g ( $k=1,96$ ;  $P=95\%$ ), respectivamente. Foram observadas seis fontes de todos os contribuintes de incertezas que afetaram a medição. **Discussão:** O valor da incerteza padrão do tipo A foi 3 vezes menor do que a incerteza padrão do tipo B para ambos os analitos. Os resultados mostram que a incerteza padrão do tipo B é um dos principais contribuintes, e o valor da incerteza expandida do método validado não mudará de teste para teste nas mesmas condições laboratoriais. **Conclusões:** A metodologia descrita neste trabalho apresenta os detalhes e aspectos práticos da abordagem híbrida usando os dados de validação do método e propõe instruções passo a passo para avaliar a incerteza de medição do método quantitativo.

**Palavras-chave:** Incerteza de Medição, HPLC, Ácido Ursólico, Ácido Oleanólico

## ABSTRACT

**Background:** Apple pomace represents a low-cost and rich source of bioactive compounds with valuable properties - ursolic acid (UA) and oleanolic acid (OA). Due to the wide range of applications in pharmaceutical and nutraceutical industries, these compounds have a high commercial value, and possessing a suitable analytical method with measurement uncertainty is of great significance and practicability. **Aim:** The purpose of the present work was to estimate detailed measurement uncertainty for the validated HPLC method combined with the extraction procedure for the determination of UA and OA in apple pomace. **Methods:** The chromatographic analysis using LC-20AD Prominence Shimadzu System and ultrasound-assisted extraction using the ultrasonic bath DW-5200DTS were performed to obtain and determine UA and OA in apple pomace. The process of measurement uncertainty evaluation was performed by the Ishikawa diagram and a combination

of bottom-up and top-down approaches. **Results:** The content of UA and OA (mg/g) in apple pomace with the value of the expanded uncertainty was calculated, which is  $7.06 \pm 0.647$  mg/g ( $k=1.96$ ;  $P=95\%$ ) and  $4.70 \pm 0.422$  mg/g ( $k=1.96$ ;  $P=95\%$ ), respectively. Six sources of all the contributors of uncertainties were observed that affected the measurement. **Discussion:** The A-type standard uncertainty value was 3 times less than the B-type standard uncertainty for both analytes. The results show that B-type standard uncertainty is a major contributor, and the value of the expanded uncertainty of the validated method will not change from test to test in the same laboratory conditions. **Conclusions:** The methodology described in this work explains well the details and practical aspects of the hybrid approach using the method validation data and proposes step-by-step instructions to evaluate the measurement uncertainty of the quantitative method.

**Keywords:** Measurement Uncertainty, HPLC, Ursolic Acid, Oleanolic Acid

## რეზიუმე

**შესავალი:** ვაშლის ნარჩენი წარმოადგენს ძვირფასი თვისებების მქონე ბიოლოგიურად აქტიური ნივთიერებების - ურსოლისა და ოლეანოლის მჟავების იაფფასიან და მდიდარ წყაროს. ფარმაცევტულ და ნუტრაცევტულ ინდუსტრიაში მათი ფართო გამოყენების გამო აღნიშნულ ნივთიერებებს მაღალი კომერციული მნიშვნელობა გააჩნიათ და სათანადო ანალიზური მეთოდის ფლობას გაზომვის განუსაზღვრელობით დიდი პრაქტიკული მნიშვნელობა გააჩნია. **მიზანი:** წარმოადგენილი ნაშრომის მიზანს წარმოადგენს ვაშლის ნარჩენში ურსოლისა და ოლეანოლის მჟავების ექსტრაქციის პროცედურასთან შეუდლებული მაღალეფექტური სითხური ქრომატოგრაფიული ვალიდირებული მეთოდის გაზომვის განუსაზღვრელობის დეტალური შეფასება. **მეთოდები:** ქრომატოგრაფიული ანალიზი და ულტრაბერითი ექსტრაქცია განხორციელდა LC-20AD Prominence Shimadzu სისტემისა და ულტრაბერითი აბაზანის DW-5200DTS გამოყენებით. გაზომვის განუსაზღვრელობის შეფასების პროცესი განხორციელდა იმიკავას დიაგრამის საშუალებით და ქვემოდან ზემოთ და ზემოდან ქვემოთ მიდგომების გაერთიანებით. **შედეგები:** გამოთვლილი იქნა ვაშლის ნარჩენში ურსოლისა და ოლეანოლის მჟავების შემცველობა (მგ/გ) გაფართოებული განუსაზღვრელობის მნიშვნელობით, რომელიც შეადგენს  $7.06 \pm 0.647$  მგ/გ ( $k=1.96$ ;  $P=95\%$ ) და  $4.70 \pm 0.422$  მგ/გ ( $k=1.96$ ;  $P=95\%$ ), შესაბამისად. გამოვლინდა განუსაზღვრელობაში მონაწილე ფაქტორების ექვსი წყარო, რომლებმაც გავლენა მოახდინა გასაზომზე. **განსჯა:** A ტიპის სტანდარტული განუსაზღვრელობა 3-ჯერ ნაკლებია, ვიდრე B ტიპის სტანდარტული განუსაზღვრელობა ორივე საანალიზო ნივთიერებისთვის. შედეგებმა აჩვენებს, რომ B ტიპის სტანდარტული განუსაზღვრელობა არის მთავარი მონაწილე და ვალიდირებული მეთოდის გაფართოებული განუსაზღვრელობის სიდიდე არ შეიცვლება იდენტური პირობებში ანალიზიდან ანალიზამდე. **დასკვნები:** ამ კვლევაში აღწერილი მეთოდოლოგია კარგად ხსნის მეთოდის ვალიდაციის მონაცემების გამოყენებით ჰიბრიდული მიდგომის დეტალურსა და პრაქტიკულ ასპექტებს და გვთავაზობს ნაბიჯ-ნაბიჯ ინსტრუქციას როგორ შევაფასოთ რაოდენობრივი განსაზღვრის მეთოდის გაზომვის განუსაზღვრელობა.

**საკვანძო სიტყვები:** გაზომვის განუსაზღვრელობა, მესქ, ურსოლის მჟავა, ოლეანოლის მჟავა

## 1. INTRODUCTION

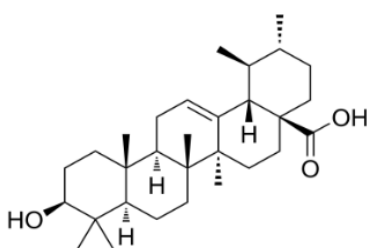
Apple pomace, as a waste material of the apple processing industry containing approximately 25% of the processed apple, represents a low-cost and rich source of fruit-derived bioactive compounds with valuable properties, including pentacyclic triterpenoids regioisomeric triterpene acids - ursolic acid (UA) and oleanolic acid (OA). These bioactive compounds have attracted much attention due to their unique and strong biological, a wide variety of approved pharmacological activities, including anti-cancer, chemopreventive, hepatoprotective, antiviral, antibacterial, anti-inflammatory,

anticoagulant, anticardiovascular, antiatherosclerotic, antidiabetic, antioxidant, immunomodulatory and gastroprotective properties. UA and OA are also utilized in preparing food supplements and important ingredients of cosmetic formulations and sports supplements. It has been reported that UA can stimulate muscle growth and enhance the epidermal permeability barrier recovery in the skin. The UA and OA chemical structures are given in Figures 1 and 2, respectively (Rubashvili *et al.*, 2020; Liese *et al.*, 2015; Khwaza *et al.*, 2020; Jin *et al.*, 2016; Woźniak *et al.*, 2015; Kashyap *et al.*, 2016; Alvarado *et al.*, 2015). Due to the wide range of applications in pharmaceutical and nutraceutical industries, these bioactive

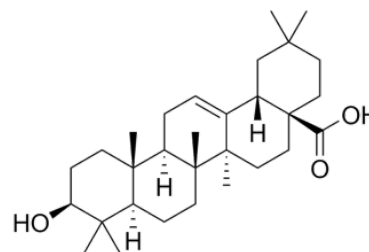
compounds have a high commercial value. Therefore, an efficient, selective, and high-yield extraction to obtain UA and OA from raw plant materials and quantitative determination of these compounds in the mentioned material and the extracted product using a suitable analytical method has great significance and practicability. Consequently, there is a need to develop and validate a new, reliable, and suitable method obtained with the combination of an extraction procedure to isolate UA and OA from apple processing waste material as a dry powdered form and an analytical procedure for the quantitative determination of these analytes in the mentioned material.

The authors of the present paper have developed a new, selective, reproducible, and high-yield extraction method by ultrasound-assisted technique for obtaining UA and OA from apple pomace and an effective, specific, sensitive, and rapid high-performance liquid chromatography (HPLC) analytical procedure to determine these target compounds quantitatively. Based on both procedures, a new combined method has been developed and validated (Rubashvili *et al.*, 2020). Analytical results are not complete unless their measurement uncertainty accompanies them. Measurement uncertainty of the analytical method may originate from many possible sources, including sample preparation, matrix effects, purity of chemical reference substances, method validation, and uncertainty associated with the analytical instrument calibration (Jebali *et al.*, 2020).

The ISO/IEC 17025 requirements for evaluation of measurement uncertainty are fulfilled if the results are obtained by following the described analytical procedure and reporting instructions, provided that all uncertainty contributors are under control (testing is performed by qualified personnel using suitable reference standards and calibrated/qualified equipment, system suitability criteria are satisfied and the repeatability is evaluated against pre-defined acceptance criteria) (OMCL guideline).



**Figure 1.** The chemical structure of UA.



**Figure 2.** The chemical structure of OA.

For consistent interpretation of the measurement results, it is necessary to evaluate the confidence that can be placed in the presentation of an analytical result, which the indication of the data quality must accompany. Therefore, method validation is an essential component of the measures, and the laboratory should implement it to produce reliable analytical data. Besides common method performance characteristics obtained in the validation process, testing laboratories shall have and apply procedures for estimating the uncertainty of measurements. This means that the analytical result cannot be viewed only as a separate value (ISO/IEC 17025; Ellison *et al.*, 2012; Rubashvili and Tsitsishvili, 2015; Senila *et al.*, 2014).

The measurement uncertainty is estimated mainly by a top-down or bottom-up approach. In the top-down approach, the major sources of uncertainty are identified and evaluated, while in the bottom-up approach, all the uncertainty sources are systematically evaluated, and only those with significant contributions are used to derive the measurement uncertainty. The top-down approach is time-consuming and requires extensive knowledge of the analytical procedure, but it enables the identification of significant uncertainty sources and, consequently, the reduction of total measurement uncertainty. Another relatively quick and easy way of uncertainty estimation is the in-house validation that includes determining the method performance parameters (Senila *et al.*, 2014; Rubashvili and Tsitsishvili, 2015).

According to Eurachem/CITAC Guides, OMCL, and EA guidelines, the bottom-up approach applies to cases with limited or no method performance data. The uncertainty arising from each source is evaluated by replicate measurements and then combined using statistical processes. The top-down approach applies to cases where method performance data are available. The combined contribution to the uncertainty is estimated using method

performance data: certified reference materials, validation study data, collaborative study data (establishment of chemical reference standards or validation of new test method), proficiency testing study data, and control charts, providing that the available performance data are used for the estimation of measurement uncertainty of the selected test/method, namely: comparable precision, satisfactory performance (system suitability) and quality control results compliant with the established analytical acceptance criteria (OMCL guideline; Eurachem guide; EA guideline; Barwick and Ellison, 2000; Barwick, 2012; Eurolab Technical Report; ISO guide 98-3; JCGM 100:2008 guide; Senila *et al.*, 2014).

The purpose of the present work was to estimate detailed measurement uncertainty for the developed and validated analytical HPLC method combined with the ultrasound-assisted extraction (UAE) procedure for the quantitative determination of UA and OA in apple pomace as an apple processing agroindustrial waste material. A hybrid approach is used as the most useful and convenient method for measurement uncertainty obtained with a combination of those as mentioned earlier - bottom-up and top-down approaches.

## 2. MATERIALS AND METHODS:

### 2.1. Materials

Local apple fruit manufacturers provided Apple pomace as an apple processing waste material. The raw material was dried in a laboratory room under controlled conditions (the temperature - 20-25 °C and the relative humidity - 30-60 %) and protected from direct sunlight. The sample was ground manually to be powdered and stored in a refrigerator before extraction (Rubashvili *et al.*, 2020).

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 ethyl acetate were purchased from Sigma-Aldrich (Germany).

### 2.2. Instrumentation

The HPLC-grade purified water was prepared using Milli Q Advantage A10 purification system (France). The UAE used the dual-frequency ultrasonic bath DW-5200DTS (bath-type) (China). 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 to prepare solutions. All the measuring equipment was appropriately calibrated. The experiment was carried out in a controlled area (temperature,  $t=22\pm 3$  °C, relative humidity,  $RH=45\pm 15$  %).

### 2.3. Extraction Procedure

The ultrasound frequency was 25 kHz; the temperature was controlled at  $25\pm 2$  °C during ultrasonication; ethanol and ethylacetate were selected as non-toxic and the best extraction solvents. The two-stage UAE was carried out by adding 10 g of the powdered dried sample of apple pomace and 100 mL of solvent in a 200 mL extraction vessel equipped with a digital temperature controller for 20 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 airflow to remove the 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 50 °C 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 of ethanol and strong sodium hydroxide solution 90:10 v/v (pH~10). Then the pH value of this solution was adjusted to  $7.0\pm 0.05$  with hydrochloride acid solution, and the obtained solution was allowed to stand for 24 hours. The crystalline solid was separated from the solution through centrifugation and then dried under air flow to obtain an extracted product (Rubashvili *et al.*, 2020).

### 2.4. Analytical Procedure

The analytical procedure 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 were reported using HPLC system software (Rubashvili *et al.*, 2020).

## 2.5. Standard and Sample Preparation

The analytical standards of UA and OA were diluted in a mixture of anhydrous formic acid and methanol 2:98 v/v (diluent) as the standard solution at the concentration – 0.25 mg/mL (10 mg of standard dissolved in 50 mL of diluent). Both standard solutions were mixed 1:1 v/v, and the obtained solution was used as the standard solution at the concentration - 0.125 mg/mL of each analyte.

To prepare the test solution, the dried extracted product (approx. 10 mg) was transferred to a 50 mL volumetric flask, diluted to volume with the diluent, and mixed well. The obtained solution was filtered through a 0.45 µm PVDF microporous membrane filter.

## 2.6. Calculations

The concentration of UA/OA -  $C_s$ , mg/mL in the test solution was calculated by Equation 1:

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

Where,  $A_s$  – The peak area of UA/OA obtained with the test solution;  $A_{st}$  – The peak area of UA/OA obtained with the standard solution;  $W_{st}$  – The weight of the standard of UA/OA, mg;  $V_{st}$  – The dilution of the standard of UA/OA, mL;  $P$  – The purity of the standard of UA/OA, %.

The content of UA/OA –  $X_i$ , mg per 1 g of the dry sample of raw material (apple pomace) was calculated using Equation 2:

$$X_i = \frac{A_s \times W_{st} \times V_2 \times W_1 \times P}{A_{st} \times W_s \times W \times V_{st} \times 100} \quad (\text{Eq. 2})$$

Where  $W$  - the weight of the dry sample of the raw material (apple pomace), g.

## 2.7. Measurement Uncertainty Evaluation

The process of measurement uncertainty evaluation was performed in four steps: in the first step, the measurand was identified; in the second step, uncertainty contributors and sources were identified by the Ishikawa diagram; in the third step, the quantification of uncertainty was performed by a combination of bottom-up and top-down approaches. After that, the standard uncertainty arising from each source was evaluated by replicate measurements and method

performance data based on the method validation study (Rubashvili *et al.*, 2020; Meyer; 2003); at the last step, the combined standard uncertainty was calculated; In order to obtain an expanded uncertainty of the method, the coverage factor –  $k=1.96$  was used as a multiplier of the combined standard uncertainty at the level of confidence of 95 % for normally distributed data and  $k=1.65$  for rectangular (uniform) distribution data (OMCL guideline).

All the calculations were performed using the validated Microsoft Excel spreadsheet software.

### 2.7.1 Standard Uncertainty of the Repeatability of the Method

The standard uncertainty of the repeatability of the method, considered as A-type uncertainty of the method, was evaluated by calculating the standard deviation of the contents of UA/OA –  $X_i$ , mg per 1 g of the dry sample of apple pomace (for 6 individual determinations). The content of each analyte was calculated using Equation 2.

The standard uncertainty –  $u_A$  of the repeatability of the method (A-type uncertainty) by Equation 3:

$$u_A = \frac{SD}{\sqrt{n}} \quad (\text{Eq. 3})$$

Where  $n$  – is the number of individual determinations;  $SD$  – the standard deviation of the contents of UA/OA in the apple pomace, mg/g.

In order to calculate the expanded uncertainty, the normal distribution was checked with the calculation of d-criteria using Equations 4, and 5:

$$s^* = \sqrt{\frac{1}{n} \sum_{i=1}^n (X_i - X)^2} \quad (\text{Eq. 4})$$

$$d = \frac{\sum_{i=1}^n |X_i - X|}{n \times S^*} \quad (\text{Eq. 5})$$

Where  $S^*$  – the dispersion value; the d-criteria of the normal distribution should be from 0.7153 to 0.9073 for six individual determinations ( $n=6$ ).

### 2.7.2 Standard Uncertainty of Standard Preparation

The combined relative standard uncertainty of standard preparation –  $u_{st}/X$  was

calculated as follows:

The relative standard uncertainty of analytical balance - Equation 6 calculated  $u(W_{St})/W_{St}$  with rectangular distribution:

$$\frac{u(W_{St})}{W_{St}} = \frac{\Delta_B}{W_{St} \times \sqrt{3}} \quad (\text{Eq. 6})$$

where  $\Delta_B$  - the standard uncertainty of the analytical balance from the calibration certificate;  $W_{St}$  - the standard UA/OA weight, mg.

The relative standard uncertainty of the standard purity  $u(P_{St})/P_{St}$  was calculated using Equation 7:

$$\frac{u(P_{St})}{P_{St}} = \frac{\Delta_P}{P_{St} \times \sqrt{3}} \quad (\text{Eq. 7})$$

Where  $\Delta_P$  - the standard uncertainty of analytical standard of UA/OA from the quality certificate;  $W_{St}$  - the weight of standard of UA/OA, mg.

The relative standard uncertainty of the molar mass of UA/OA (Molecular formula:  $C_{30}H_{48}O_3$ )  $u(M)/M$  was calculated using Equation 8:

$$\frac{u(M)}{M} = \frac{\sqrt{30 \times 0.0006^2 + 3 \times 0.00021^2 + 48 \times 0.0000784^2}}{456.7 \times \sqrt{3}} \quad (\text{Eq. 8})$$

Where  $M$  - is the molar mass of UA/OA, 456.7 g/mol.

The relative standard uncertainty of the mass of standard -  $u(m_{St})/W_{St}$  was calculated by Equation 9:

$$\frac{u(m_{St})}{W_{St}} = \sqrt{\left(\frac{u(W_{St})}{W_{St}}\right)^2 + \left(\frac{u(P_{St})}{P_{St}}\right)^2 + \left(\frac{u(M)}{M}\right)^2} \quad (\text{Eq. 9})$$

The relative standard uncertainty of the used volumetric glassware - Equation 10 calculated  $u(V_{St}G)/V_{St}i$  with triangular distribution:

$$\frac{u(V_{St}G)i}{V_{St}i} = \frac{\Delta_V}{V_{St}i \times \sqrt{6}} \quad (\text{Eq. 10})$$

Where  $\Delta_V$  - the standard uncertainty of the glassware from the calibration certificate, mL;  $V_{St}$  - the measured dilution volume of standard, mL.

The relative standard uncertainty of the temperature effect of the volumetric glassware -  $u(V_{St}T)i/V_{St}i$  with rectangular distribution was calculated by Equation 11:

$$\frac{u(V_{St}T)i}{V_{St}i} = \frac{V_E}{V_{St}i \times \sqrt{3}} \quad (\text{Eq. 11})$$

Where  $V_T$  - the expansion of the volume, mL was calculated by Equation 12:

$$\Delta_V = V_{St}i \times \Delta t \times \frac{0.00021}{1} \quad (\text{Eq. 12})$$

Where,  $\Delta t$  - the half value of the temperature range in the laboratory room, °C.

The relative standard uncertainty of the dilution volume of standard -  $u(V_{St})i/V_{St}i$  was calculated by Equation 13:

$$\frac{u(V_{St})i}{V_{St}i} = \sqrt{\left(\frac{u(V_{St}G)i}{V_{St}i}\right)^2 + \left(\frac{u(V_{St}T)i}{V_{St}i}\right)^2} \quad (\text{Eq. 13})$$

The combined relative standard uncertainty of standard preparation expressed as the uncertainty of the concentration of UA/OA in standard solution -  $u_{St}/X$  was calculated by Equation 14:

$$\frac{u_{St}}{X} = \sqrt{\left(\frac{u(m_{St})}{W_{St}}\right)^2 + \left(\sum_{i=1}^k \frac{u(V_{St})i}{V_{St}i}\right)^2} \quad (\text{Eq. 14})$$

### 2.7.3 Standard Uncertainty of Sample Preparation

The combined relative standard uncertainty of sample preparation expressed as the uncertainty of the concentration of UA/OA in test solution -  $u_s/X$  was calculated as follows:

The relative standard uncertainty of the repeatability of weighting -  $u(W_{rep})/W_s$  with rectangular distribution was calculated using Equation 15:

$$\frac{u(W_{rep})}{W_s} = \frac{SD}{W_s \times \sqrt{3}} \quad (\text{Eq. 15})$$

Where  $SD$  - the standard deviation of the extracted product weights ( $n=6$ ), mg, which was calculated using Equation 16:

$$SD = \sqrt{\frac{1}{(n-1)} \sum_{j=1}^n (W_i - W_s)^2} \quad (\text{Eq. 16})$$

Where,  $W_i$  - the weight of each sample of the extracted product, mg.

The relative standard uncertainty of analytical balance - Equation 17 calculated  $u(W_s)/W_s$  with rectangular distribution:

$$\frac{u(W_s)}{W_s} = \frac{\Delta_B}{W_s \times \sqrt{3}} \quad (\text{Eq. 17})$$

The relative standard uncertainty of the mass of the sample of the extracted product -  $u(m_s)/W_s$  was calculated using Equation 18:

$$\frac{u(m_s)}{W_s} = \sqrt{\left(\frac{u(W_s)}{W_s}\right)^2 + \left(\frac{u(W_{rep})}{W_s}\right)^2} \quad (\text{Eq. 18})$$

The relative standard uncertainty of the used volumetric glassware - Equation 19 calculated  $u(V_s G)/V_{si}$  with triangular distribution:

$$\frac{u(V_s G)_i}{V_{si}} = \frac{\Delta_V}{V_{si} \times \sqrt{6}} \quad (\text{Eq. 19})$$

Where  $\Delta_V$  - the standard uncertainty of the glassware from the calibration certificate, mL;  $V_s$  - the measured dilution volume of the extracted product, mL sample.

The relative standard uncertainty of the temperature effect of the volumetric glassware -  $u(V_s T)_i/V_{si}$  with rectangular distribution was calculated by Equation 20:

$$\frac{u(V_s T)_i}{V_{si}} = \frac{V_E}{V_{si} \times \sqrt{3}} \quad (\text{Eq. 20})$$

Where  $V_T$  - the expansion of the volume, mL was calculated by Equation 21:

$$\Delta_V = V_{si} \times \Delta t \times \frac{0.00021}{1} \quad (\text{Eq. 21})$$

Where,  $\Delta t$  - the half value of the temperature range in the laboratory room, °C.

The relative standard uncertainty of the

dilution volume of the sample -  $u(V_s)_i/V_{si}$  using Equation 22:

$$\frac{u(V_s)_i}{V_{si}} = \sqrt{\left(\frac{u(V_s G)_i}{V_{si}}\right)^2 + \left(\frac{u(V_s T)_i}{V_{si}}\right)^2} \quad (\text{Eq. 22})$$

The combined relative standard uncertainty of sample preparation - Equation 23 calculated  $u_s/X$ :

$$\frac{u_s}{X} = \sqrt{\left(\frac{u(m_s)}{W_s}\right)^2 + \left(\sum_{i=1}^k \frac{u(V_s)_i}{V_{si}}\right)^2} \quad (\text{Eq. 23})$$

#### 2.7.4 Standard Uncertainty of the mass of the apple pomace

The combined relative standard uncertainty of the mass of the apple pomace -  $u(m)/W$  was calculated as follows:

The relative standard uncertainty of the repeatability of weighting balance - the Equation 24 calculated  $u(W_{rep})/W$  with rectangular distribution:

$$\frac{u(W_{rep})}{W} = \frac{SD}{W \times \sqrt{3}} \quad (\text{Eq. 24})$$

Where SD - the standard deviation of the weights of the apple pomace samples ( $n=6$ ), g, which was calculated using Equation 25:

$$SD = \sqrt{\frac{1}{(n-1)} \sum_{j=1}^n (W_i - W)^2} \quad (\text{Eq. 25})$$

The relative standard uncertainty of analytical balance - Equation 26 calculated  $u(W)/W$  with rectangular distribution:

$$\frac{u(W)}{W} = \frac{\Delta_B}{W \times \sqrt{3}} \quad (\text{Eq. 26})$$

The combined relative standard uncertainty of the mass of the apple pomace -  $u(m)/W$  was calculated using Equation 27:

$$\frac{u(m)}{W} = \sqrt{\left(\frac{u(W_{rep})}{W}\right)^2 + \left(\frac{u(W)}{W}\right)^2} \quad (\text{Eq. 27})$$

#### 2.7.5 Measurement Uncertainty of the Accuracy of the Method

In order to evaluate the standard uncertainty of the accuracy of the method -  $u_R$ , the

mean recovery value – R (R, %/100) and the relative standard deviation (RSD, %) of the percentage recoveries (n=3) were used based on the accuracy study of the combined method (Rubashvili *et al.*, 2020). Equation 28 calculated the standard uncertainty of the accuracy of the combined method:

$$u_R = \frac{RSD}{100 \times \sqrt{3}} \quad (\text{Eq. 28})$$

### 2.7.6 Standard Uncertainty of Analytical Equipment - HPLC

The combined relative standard uncertainty of analytical equipment – HPLC system –  $u_E/A$  was calculated with rectangular distribution was calculated using Equation 29:

$$\frac{u(E)}{A} = \sqrt{\left(\frac{\Delta E}{A_{St} \times \sqrt{3}}\right)^2 + \left(\frac{\Delta E}{A_S \times \sqrt{3}}\right)^2} \quad (\text{Eq. 29})$$

Where  $\Delta E$  - is the standard uncertainty of the HPLC system from the calibration certificate.

### 2.7.7 Combined Standard Uncertainty and Expanded Uncertainty of the Method

Combined B-type standard uncertainty –  $u_B$  was calculated using Equation 30:

$$u_B = X \times \sqrt{\left(c_1 \times \frac{u_{St}}{X}\right)^2 + \left(c_2 \times \frac{u_S}{X}\right)^2 + \left(c_3 \times \frac{u(m)}{W}\right)^2 + \left(c_4 \times u_R\right)^2 + \left(c_5 \times \frac{u(E)}{A}\right)^2} \quad (\text{Eq. 30})$$

Where  $c_i$  - the sensitivity coefficient was considered equal to 1 (Eurachem guide).

Equation 31 calculated the combined standard uncertainty of the combined method -  $u$ :

$$u = \sqrt{u_A^2 + u_B^2} \quad (\text{Eq. 31})$$

Equation 32 calculated the expanded uncertainty of the combined method -  $U$ :

$$U = u \times k \quad (\text{Eq. 32})$$

Where  $k$  - the coverage coefficient equals 1.96 for normal distribution and 1.65 for rectangular distribution, based on the calculated results of d-criteria for both analytes.

## 3. RESULTS AND DISCUSSION:

The measurement of the method was the content of UA/OA in the apple processing waste material (apple pomace), expressed in mg/g calculated by Equation 2. Each parameter that affects the value of the measurand is shown as a cause and effect Ishikawa diagram (Figure 3) (OMCL guideline; Ellison and Barwick, 1998).

### 3.1. Results

The first source affected the measurand – the content of UA/OA in apple pomace (mg/g) was uncertainty arising from the repeatability of the method, which was carried out by preparing and injecting a standard solution with six replicates and six test solutions into the HPLC system by the validated method. Based on the obtained analytical data, the contents of UA/OA –  $X_i$ , mg/g in the samples of the apple pomace were calculated using Equation 2; Then, the standard deviation (n=6) of the contents of each analyte was used to evaluate the standard uncertainty of the repeatability of the method –  $u_A$  (A-type uncertainty) using Equation 3. The obtained results are given in Table 1.

**Table 1.** The results of the repeatability and A type standard uncertainty

№	The content of UA in apple pomace, mg/g	The content of OA in apple pomace, mg/g
1	6.93	4.49
2	7.39	4.64
3	6.86	4.71
4	7.34	4.99
5	7.01	4.74
6	6.83	4.66
<b>Average</b>	<b>7.06</b>	<b>4.70</b>
<b>SD</b>	<b>0.24</b>	<b>0.16</b>
<b>n</b>	<b>6</b>	<b>6</b>
<b><math>u_A</math></b>	<b>0.099</b>	<b>0.067</b>
<b>d-Criteria</b>	<b>0.9019</b>	<b>0.8990</b>

The second source that affected the measurand was uncertainty arising from standard preparation, which was calculated by the combined relative standard uncertainty of standard preparation –  $u_{st}/X$  (Equation 14). This source includes the following contributors: 1) the



relative standard uncertainty of analytical balance -  $u(W_{St})/W_{St}$  (Equation 6); 2) the relative standard uncertainty of the standard purity  $u(P_{St})/P_{St}$  (Equation 7); 3) the relative standard uncertainty of the molar mass of UA/OA -  $u(M)/M$  (Equation 8); 4) the relative standard uncertainty of the mass of standard -  $u(m_{St})/W_{St}$  (Equation 9); 5) the relative standard uncertainty of the used volumetric glassware -  $u(V_{St}G)/V_{St}i$  (Equation 10); 6) the relative standard uncertainty of the temperature effect of the volumetric glassware -  $u(V_{St}T)i/V_{St}i$  (Equation 13). The obtained results of the combined relative standard uncertainty of standard preparation are given in Table 2.

The third source was evaluated by the combined relative standard uncertainty of sample preparation -  $u_s/X$ , which was calculated using Equation 23. This contains the following contributors: 1) the relative standard uncertainty of the repeatability of weighting -  $u(W_{rep})/W_s$  (Equation 15); 2) the relative standard uncertainty of analytical balance (Equation 17); 3) the relative standard uncertainty of the mass of the sample of the extracted product -  $u(m_s)/W_s$  (Equation 18); The relative standard uncertainty of the dilution volume of the sample -  $u(V_s)i/V_{si}$  (Equation 22). The results are given in Table 3.

The combined relative standard uncertainty of the mass of the apple pomace -  $u(m)/W$  calculated using Equation 27 was one of the determined main sources that affected the measurand. The source combines two contributors: 1) the relative standard uncertainty of the repeatability of weighting balance  $u(W_{rep})/W$  (Equation 24) and 2) the relative standard uncertainty of analytical balance  $u(W)/W$  (Equation 26). The results are shown in Table 4.

The accuracy of the method was contributed to the value of the expanded uncertainty of the method. The standard uncertainty of the accuracy of the method -  $u_R$  was calculated using Equation 28 and is shown in Table 5.

**Table 5.** The results of the accuracy of the method

Parameter	UA	OA
R, %	96.85	4.70
RSD, % (n=3)	1.75	0.53
$u_R$	0.01	0.003

The last source that affected the

measurand was uncertainty arising from analytical equipment – HPLC system. This source was evaluated by calculating the combined relative standard uncertainty of analytical equipment –  $u_E/A$  (Equation 29). The obtained results are given in Table 6.

**Table 7.** The values of standard uncertainties and expanded uncertainty of the combined method expressed in mg/g

Parameter	UA	OA
$u_A$	0.099	0.067
$u_B$	0.315	0.205
$u$	0.330	0.215
$k$	1.96	1.96
$U$	0.647	0.422

The values of the combined B-type standard uncertainty –  $u_B$ , the combined standard uncertainty –  $u$ , and the expanded uncertainty of the method –  $U$  calculated using Equations 30, 31, 32, respectively, are shown in Table 7. Based on the calculated results of d-criteria for both analytes, normal distribution of analytical data appeared in both cases. Accordingly, 1.96 was used as the coverage coefficient –  $k$  to calculate the expanded uncertainty value.

The content of each test compound expressed in mg per 1 g of the dry sample of the agroindustrial waste material (apple pomace) was calculated. The obtained results indicate that the content of UA and OA, mg/g in apple pomace varies from 6.86 to 7.39 mg/g and from 4.49 to 4.99 mg/g, respectively; the average content of UA and OA with the value of the expanded uncertainty is  $7.06 \pm 0.647$  mg/g ( $k=1.96$ ;  $P=95\%$ ) and  $4.70 \pm 0.422$  mg/g ( $k=1.96$ ;  $P=95\%$ ), respectively.

### 3.2. Discussion

The measurement uncertainty of the method includes all the uncertainties arising from each individual source and contributor determined by the cause and effect Ishikawa diagram and affected the measurand – the content of UA and OA in apple pomace expressed in mg per 1 g. There are observed six sources of all the contributors: 1) uncertainty of standard preparation; 2) uncertainty of sample preparation; 3) uncertainty of the accuracy of the method; 4) uncertainty of the repeatability of the method; 5) uncertainty of the mass of apple pomace sample,

and 6) uncertainty of the analytical equipment. Each source was evaluated, and the results show that all the contributors contributed to the combined standard uncertainty arising from both extraction and chromatographic analytical procedures. All the uncertainty sources equally affect the measurement of the method. The A-type standard uncertainty value was 3 times less than the B-type standard uncertainty for both analytes. The results show that B-type standard uncertainty is a major contributor and equals approximately 95 % of the combined standard uncertainty for both analytes. Therefore, the value of the expanded uncertainty of the validated method will not change from test to test in the same laboratory conditions by using the same equipment, instruments, and reagents during the routine intra-day or inter-day analyses, which confirms the suitability and robustness of the validated method.

#### 4. CONCLUSIONS:

The hybrid approach used in the measurement uncertainty of the analytical method fully ensures a detailed assessment of uncertainty and consideration of all contributors. Furthermore, it is possible to reliably use the value of the expanded uncertainty calculated by this approach based on the method validation data in routine analyses so that the measurement uncertainty is not needed to calculate for each routine analysis. The presented work explains well the details and practical aspects of this approach and proposes the step-by-step methodology of measurement uncertainty according to Eurachem and EA guidelines which can be used to evaluate measurement uncertainty for other analytical HPLC methods. The proposed validated method with measurement uncertainty can be applied successfully to control ursolic and oleanolic acids in apple peel, apple pomace, any apple processing agroindustrial waste material, and the dry extracted product obtained from apple pomace.

#### 5. DECLARATIONS

##### 5.1. Study Limitations

The study is limited to the sample size and methods utilized, and no further limitations were known at the time of the study.

##### 5.2. Funding source

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#### 5.3. Competing Interests

The authors declare that there are no conflicts of interest in this publication.

#### 5.5. Open Access

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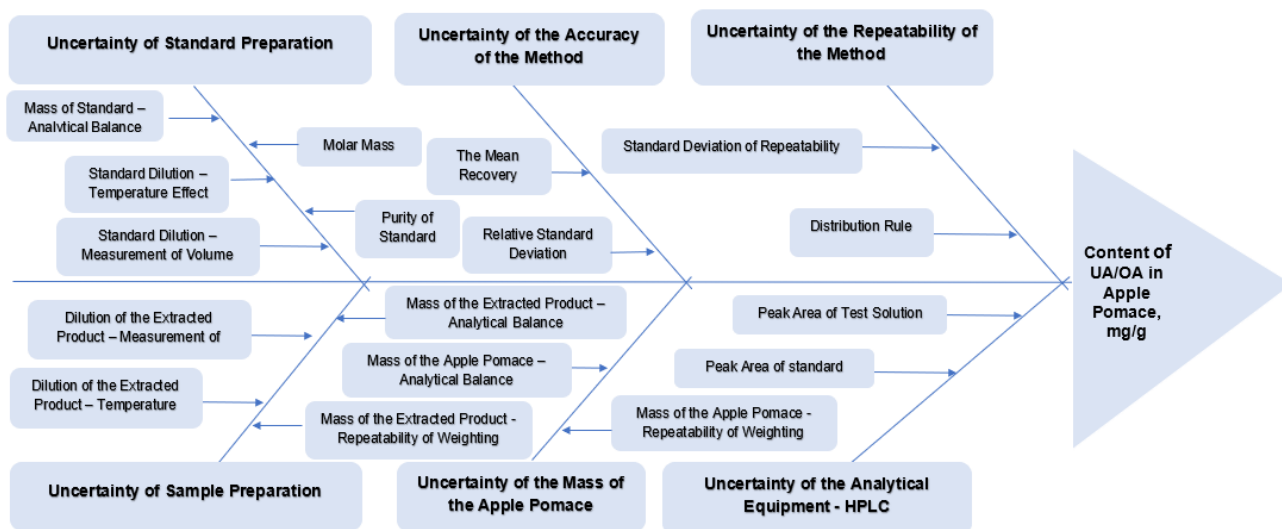
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**Figure 3.** The cause and effect ishikawa diagram.

**Table 2.** The budget of evaluation of measurement uncertainty of standard preparation

Nº	Contributor	Value	Type	Unit	Relative Standard Uncertainty of Contributor	Distribution	Sensitivity Coefficient	Relative Standard Uncertainty	
<b>Ursolic Acid</b>									
1	Mass of Standard - Analytical Balance - $u(W_{St})/W_{St}$	10.5	B	mg	0.00055	Rectangular	1	0.00121	
2	Purity of Standard - $u(P_{St})/P_{St}$	96	B	%	0.00107	Rectangular	1		
3	Molar Mass - $u(M)/M$	456.7	B	g/mol	0.000004	Rectangular	1		
4	Dilution of Standard - Measurement of Volume - $u(V_{StG})/V_{Si}$	50	B	mL	0.00049	Triangular	1		0.00576
5	Dilution of Standard - Temperature Effect - $u(V_{StT}i)/V_{Si}$	50	B	mL	0.00036	Rectangular	1		
6	Dilution of Standard - Measurement of Volume - $u(V_{StG})/V_{Si}$	5	B	mL	0.000572	Triangular	1		
7	Dilution of Standard - Temperature Effect - $u(V_{StT}i)/V_{Si}$	5	B	mL	0.00036	Rectangular	1		
<b>Combined relative standard uncertainty – <math>u_{St}/X</math></b>								0.00588	
<b>Oleanolic Acid</b>									
1	Mass of Standard - Analytical Balance - $u(W_{St})/W_{St}$	12.5	B	mg	0.00046	Rectangular	1	0.00088	
2	Purity of Standard - $u(P_{St})/P_{St}$	98	B	%	0.00075	Rectangular	1		
3	Molar Mass - $u(M)/M$	456.7	B	g/mol	0.000004	Rectangular	1		
4	Dilution of Standard - Measurement of Volume - $u(V_{StG})/V_{Si}$	50	B	mL	0.00049	Triangular	1		0.00576
5	Dilution of Standard - Temperature Effect - $u(V_{StT}i)/V_{Si}$	50	B	mL	0.00036	Rectangular	1		
6	Dilution of Standard - Measurement of Volume - $u(V_{StG})/V_{Si}$	5	B	mL	0.000572	Triangular	1		
7	Dilution of Standard - Temperature Effect - $u(V_{StT}i)/V_{Si}$	5	B	mL	0.00036	Rectangular	1		
<b>Combined relative standard uncertainty – <math>u_{St}/X</math></b>								0.00583	

**Table 3.** The budget of evaluation of measurement uncertainty of sample preparation

No	Contributor	Value	Type	Unit	Relative Standard Uncertainty of Contributor	Distribution	Sensitivity Coefficient	Relative Standard Uncertainty
<b>Ursolic Acid</b>								
1	Mass of Sample - Analytical Balance - $u(W_s)/W_s$	11.8	B	mg	0.00049	Rectangular	1	0.04329
2	Repeatability of Weighting - $u(W_{rep})/W_s$	11.8	A	mg	0.043328	Rectangular	1	
3	Dilution of Sample - Measurement of Volume - $u(V_sG)/V_{si}$	50	B	mL	0.00049	Triangular	1	0.00061
4	Dilution of Sample - Temperature Effect - $u(V_{stT})i/V_{si}$	50	B	mL	0.00036	Rectangular	1	
<b>Combined relative standard uncertainty – <math>u_s/X</math></b>								0.04329
<b>Oleanolic Acid</b>								
1	Mass of Sample - Analytical Balance - $u(W_s)/W_s$	11.8	B	mg	0.00049	Rectangular	1	0.04329
2	Repeatability of Weighting - $u(W_{rep})/W_s$	11.8	A	mg	0.043328	Rectangular	1	
3	Dilution of Sample - Measurement of Volume - $u(V_sG)/V_{si}$	50	B	mL	0.00049	Triangular	1	0.00061
4	Dilution of Sample - Temperature Effect - $u(V_{stT})i/V_{si}$	50	B	mL	0.00036	Rectangular	1	
<b>Combined relative standard uncertainty – <math>u_s/X</math></b>								0.04329

**Table 4.** The budget of evaluation of measurement uncertainty of the mass of apple pomace

No	Contributor	Value	Type	Unit	Relative Standard Uncertainty of Contributor	Distribution	Sensitivity Coefficient	Relative Standard Uncertainty
<b>Ursolic Acid</b>								
1	Mass of Sample - Analytical Balance - $u(W)/W$	10.38	B	g	0.0000006	Rectangular	1	0.00409
2	Repeatability of Weighting - $u(W_{rep})/W$	10.38	A	g	0.00409	Rectangular	1	
<b>Combined relative standard uncertainty – <math>u(m)/W</math></b>								0.00409
<b>Oleanolic Acid</b>								
1	Mass of Sample - Analytical Balance - $u(W)/W$	10.38	B	g	0.0000006	Rectangular	1	0.00409
2	Repeatability of Weighting - $u(W_{rep})/W$	10.38	A	g	0.00409	Rectangular	1	
<b>Combined relative standard uncertainty – <math>u(m)/W</math></b>								0.00409

**Table 6.** The budget of evaluation of measurement uncertainty of analytical equipment - HPLC

No	Contributor	Value	Type	Unit	Relative Standard Uncertainty of Contributor	Distribution	Sensitivity Coefficient	Relative Standard Uncertainty
<b>Ursolic Acid</b>								
1	Peak area of the standard solution - HPLC	1781997.00	B	mAU	0.00029	Rectangular	1	0.00042
2	Peak area of the test solution - HPLC	2345089.83	B	mAU	0.00029	Rectangular	1	
<b>Combined relative standard uncertainty – <math>u_{\epsilon}/A</math></b>								0.00042
<b>Oleanolic Acid</b>								
1	Peak area of the standard solution - HPLC	3230243.17	B	mAU	0.00029	Rectangular	1	0.00042
2	Peak area of the test solution - HPLC	2349147.33	B	mAU	0.00029	Rectangular	1	
<b>Combined relative standard uncertainty – <math>u_{\epsilon}/A</math></b>								0.00042