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RESEARCH ARTICLE

LC-MS method for the detection and quantification of ursolic acid and uvaol levels in olive leaves and oregano

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ABSTRACT

Ursolic acid and uvaol belong to pentacyclic triterpenes are both secondary metabolites with significant biological activity. Ursolic acid and uvaol are exist in many natural matrices such as olive leaves, oregano and thymus. Therefore, a new rapid and validated UPLC method was developed to identify and quantify ursolic acid and uvaol in dry olive leaves (*Olea europaea* L.) planted in Kuwait and dry oregano (*Origanum vulgare* L.) planted in Palestine and Spain. The separation of ursolic acid and uvaol was achieved by using BEH Phenyl (1.7 µm, 2.1 x 100 mm) analytical column (Waters® Acquity UPLC) and a mobile phase composed of water and acetonitrile (37:63 v/v). Solid-phase extraction cartridges (HyperSep™) was used to extract ursolic acid and uvaol from natural matrices. Ursolic acid and uvaol were detected in all-natural matrices used. The concentrations of ursolic acid and uvaol in olive leaves were 55.10 µg/g ± 0.72 and 314.29 µg/g ± 0.97, respectively. In contrast, ursolic acid and uvaol concentrations in oregano brands were ranging from 25.57 µg/g ± 0.35- 28.70 µg/g ± 0.36 and 19.07 µg/g ± 0.38 - 48.10 µg/g ± 0.62, respectively. In conclusion, the developed method was capable to identify and determine ursolic acid and uvaol in pure powder and natural matrices with good linearity, accuracy and precision.

Keywords: Olive leaves; Oregano; Ursolic acid; Uvaol; LC-MS

INTRODUCTION

Ursolic acid (molecular weight (MW) 456.711 g/mol) and uvaol (MW 442.728 g/mol), as shown in Fig. 1, possess a similar chemical structure with only difference that carboxylic acid functionality of ursolic acid is replaced by alcoholic function in uvaol. Both of these substances belong to pentacyclic triterpenes, ursolic acid is a secondary metabolite and a constituent of some higher plants. In literature, ursolic acid has been investigated to have a chemo-preventive and anti-cancer effect (Novotny et al., 2001; Wozniak et al., 2015; Mancha-Ramirez and Slaga, 2016), anti-inflammatory effect (Ikeda et al., 2008) and anti-hyper-lipidemic effect (Mancha-Ramirez and Slaga, 2016; Katashima et al., 2017).

Uvaol, another secondary metabolite, is a significantly less stable substance (that is easily oxidized to a carboxylic acid – ursolic acid) and detected in a few types of plants. One of the typical sources of uvaol is olive oil (Sanchez-Quesada et al., 2013; Lou-Bonafonte et al., 2012; Giuffre, 2012), the biological or pharmacological activities of uvaol contributes in the general positive biological effects of uvaol. Due to uvaol instability, it is rarely investigated on its own. However, its anti-inflammatory and anti-oxidative properties were confirmed (Agra et al., 2016).

In the present work, UPLC was applied to simultaneously detect and quantify two secondary metabolites – ursolic acid and uvaol - in natural origin matrices with a relatively high content of uvaol (dried olive leaves) and with a very low concentration of uvaol (dry oregano) to prove the versatility of this method. Previously, simultaneous detection of ursolic acid and uvaol was performed using various high-performance chromatography methods (Cayuela et al., 2006; Gimenez et al., 2015; Cefarelli et al., 2006) and UPLC was applied to determine ursolic acid (Y. Xia et al., 2011; Cao et al., 2016; Pandey et al., 2015;

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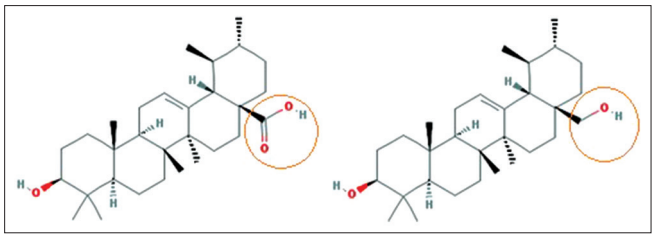

Fig 1. Chemical structures of ursolic acid (A) and uvaol (B).

Zhang et al., 2011). However, to the best of our knowledge, UPLC determination of ursolic acid and uvaol has not been reported in scientific literature. Although, some official bodies, including European Union, have recommended the use of tandem thin-layer chromatography and gas chromatography (TLC-GC) to determine uvaol in olive oil (Commission Regulation (EEC), 1991), in our study we have established a rapid, accurate and validated UPLC method to detect and quantify ursolic acid and uvaol in natural resources, as an alternative analytical technique to TLC-GC method.

MATERIAL AND METHODS

Materials

Ursolic acid and uvaol standards were from Sigma-Aldrich (Dorset, UK). Dry oregano samples (*Origanum vulgare* L.*)* were purchased locally as products of two different companies (Albarak; the origin of the product: Palestine; Waitro**'s**; origin of the product: Spain). Olive leaves were picked from olive plant obtained from a local planting shop (cultivated in Kuwait). High performance liquid chromatography (HPLC) grade acetonitrile was provided by Sigma Aldrich (Dorset, UK). HPLC grade water was prepared "in house" with a MilliQfilter (Millipore, Watford, UK). HyperSep™ phenyl solid-phase extraction cartridges purchased from ThermoFisher Scientific (Rugby, Canada). Nylon solvent filters (0.45 um) were purchased from Waters Corporation (Milford, USA). Formic acid reagent grade, ≥95% was obtained from Sigma Aldrich (Dorset, UK). Syringe membrane filters (13mm) were purchased from Kinesis Scientific Expert (Cambridgeshire, UK). Benzyl (3-fluoro-4-morpholinophenyl) carbamate was synthesized in our laboratory and was used as an internal standard in this study.

Instrumentation

UPLC Instrumentation

Waters® Acquity UPLC system with quaternary Solvent Manager (H-Class), Sample Manager and UV detector, and Waters® Acquity UPLC BEH Phenyl (1.7 µm, 2.1 x 100 mm) analytical column were used for the analysis and method validation. Empower® software was used for data processing and reporting.

Chromatographic Conditions

An isocratic mobile phase composed of 0.1% v/v formic acid in water and acetonitrile in proportion of 37:63 v/v was used and pumped at a flow rate of 0.3 mL/min. The column temperature was set at 45 ºC and samples were analyzed at a wavelength of 220 nm and were injected at 10 µL injection volume.

LC-MS system

Waters® Xevo G2-S QToF was coupled with Waters® Acquity UPLC system with quaternary Solvent Manager (H-Class) via electrospray ionization (ESI) source. The operating parameters were as follows: the sheath gas flow rate was set at 30 arbitrary unit and the auxiliary flow rate was set at 5 arbitrary unit. The voltage of the capillary was set at 4.5 V, sampling cone was 50 and source temperature was 120 °C. The desolvation temperature was set at 450 °C.

LC-MS analysis

1 mL of each extract was used for the LC-MS analysis. LC-MS was acquired using the following parameters: Flow rate was 0.3 mL/min, injection volume was 10 µL. Isocratic elution was carried out with a mobile phase of 0.1% formic acid in water and acetonitrile (37:63 v/v). The column temperature was set at 45 ºC and was used to perform the analysis of all samples. For the MS, both positive and negative polarity modes were used and the mass range was from 50-1000 mass units.

Preparation of standard stock and working solutions

A stock solution of ursolic acid and uvaol was prepared separately by dissolving 50 mg of the reference standard in 50 mL methanol in a volumetric flask to produce a concentration of 1 mg/mL. A stock solution of benzyl (3-fluoro-4-morpholinophenyl) carbamate, was prepared to be used as an internal standard by dissolving 5 mg in 5 mL of methanol to produce a concentration of 1 mg/mL. The prepared stock solutions were stored at 4 °C.

Linearity and calibration standards of the pure bulk powder

Precisely measured aliquots of ursolic acid and uvaol were relocated from their working standard solutions (1 mg/mL) into a series of 10 mL volumetric flasks and the volume completed with the mobile phase. The calibration samples consist of five concentrations of ursolic acid (10 – 400 µg/mL) and five concentrations of uvaol (15 - 400 µg/mL). 200 µL of benzyl (3-fluoro-4-morpholinophenyl) carbamate was added to each sample as an internal standard. The samples were injected separately into the BEH Phenyl column under a flow rate of 0.3 mL/min. The relative peak area of each drug was recorded against its concentration, the linearity curves were constructed and the regression equations were computed.

Linearity and calibration standards of analytes in natural sources after solid-phase extraction

Different aliquots from the standard working solutions were used to prepare calibration standards using benzyl (3-fluoro-4-morpholinophenyl) carbamate as an internal standard. Five different concentrations of ursolic acid (15 – 400 μg/mL) and five concentration of uvaol (25 - 400 μg/mL) were used as the calibration samples. Quality control samples used were: 15 μg/mL, 100 μg/mL, 200 μg/mL, 300 μg/mL and 400 μg/mL for ursolic acid; however uvaol concentrations were 25 μg/mL, 100 μg/mL, 200 μg/mL, 300 μg/mL and 400 μg/mL.

Solid-phase extraction procedure

Aliquots of 1 mL of calibration standard solutions of ursolic acid and uvaol were loaded into solid-phase extraction cartridges on a vacuum 20 position extraction manifold (Waters). The cartridges were pre-conditioned with 1 mL of methanol followed by 1 mL of water. Then, the analytes (ursolic acid and uvaol) were eluted with 800 μL of the mobile phase into a 2 mL glass vials. Afterwards, samples were filtered using syringe membrane filters (13 mm) kinesis® and 200 μL of the internal standard was added prior to analysis.

Validation

Validation of the method was performed according to The International Conference on Harmonization (ICH) guidelines (ICH, 2005, Al-Tannak and Hemdan, 2018).

System Suitability Test

A system suitability test was established from three replicate injections of a solution containing 200 μg/mL of ursolic acid and uvaol. The peak tailing for the drug was measured. A useful and practical measurement of peak shape, peak tailing, and theoretical plate count was determined. The column plate number was determined using the formula $N = 5.54 (T_R / W_h)^2$, where T_R is the peak retention time and W_h is the bandwidth at 50% of peak height.

Accuracy and precision

Accuracy and precision of the UPLC method for the combination were evaluated by preparing six sets of the mixture in the concentration ranges of the calibration curve. Accuracy and precision were performed in triplicates using three concentration levels of 10, 200 and 400 μg/mL for ursolic acid and 15, 200 and 400 for uvaol in mobile phase. Moreover, accuracy and precision were determined in triplicates using three concentration levels of 15, 200 and 400 μg/mL for ursolic acid and 25, 200 and 400 μg/mL for uvaol after solid-phase extraction. One set (n = 3 of each) of solid-phase extracted standards and one set of standards in mobile phase were prepared at room temperature (22-25 °C), while other five sets (n =3 of each) were prepared and stored at 4 °C for mixture dissolved in mobile phase samples and solid-phase extracted samples for 10 days. Percentage relative standard deviation (%RSD) and percentage deviation from the nominal concentration (%DEV) were used to calculate the intra- and inter-assay precision and accuracy.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Stock solutions of ursolic acid and uvaol were prepared at concentrations of 1–100 μg/mL. The LOD and LOQ for ursolic acid and uvaol were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively.

Extraction Recovery

Two sets of calibration standards samples containing 15 μg/mL.- 100 μg/mL - 200 μg/mL - 300 μg/mL and 400 μg/mL of ursolic acid and and 25 μg/mL - 200 μg/mL - 300 μg/mL and 400 μg/mL of uvaol were prepared in the mobile phase. All standards prepared were mixed with 200 μL of the internal standard (benzyl (3-fluoro-4-morpholinophenyl) carbamate) and extracted as mentioned previously. The extraction recoveries were estimated from the slopes of the standard curve of ursolic acid and uvaol (Table.2).

Extraction procedure from natural matrices

The leaves of olive tree were dried under room temperature for 15 days. Then the dried leaves were mechanically pulverized and one gram of pulverized leaves were placed in a conical flask and 300 mL of methanol was added to extract ursolic acid and uvaol. The mixture was then sonicated for 5 hours and left overnight. Ursolic acid and uvaol were detected and quantified in triplicates.

Table 1: Parameters of system suitability testing of the adopted chromatographic methods for the simultaneous determination of Ursolic acid and Uvaol

Parameters	Ursolic acid	Uvaol	Reference value
Resolution (R_s)	2.21	2.21	$Rs \geq 2$
Tailing factor (T)	1.13	0.85	$T \leq 2$
Capacity factor (K')	5.51	6.63	1< K'<10
Selectivity (α)	1.20	1.20	$\alpha > 1$
Asymmetry factor (Af)	1.05	1.01	0.9 < Af < 1.1
Theoretical plates (N)	4801	4101	N > 2000
Height equivalent to theoretical plate (HETP; cm plate^{-1})	0.002	0.002	The smaller the value, the higher the column efficiency

Moreover, one gram of each oregano samples (planed in Palestine and Spain) were weighed. Samples were placed in a conical flask and 300 mL of methanol was added to extract ursolic acid and uvaol. Then, the mixture was sonicated for 5 hours and left overnight. Ursolic acid and uvaol were detected and quantified in triplicates.

1 mL of each extract (olive leaves and oregano) was taken, centrifuged for 20 minutes to precipitate large particles like chlorophyll. Ursolic acid and uvaol were extracted from olive leaves and oregano by using the solid-phase extraction procedure described above. The concentration of both analytes was calculated from the computed regression equations.

RESULTS AND DISCUSSION

Various extraction cartridges, temperatures, organic modifiers and buffers with different pH, were examined for the optimization procedure during the method development. The optimum resolution and peak shape were obtained with 0.1% formic acid in water/acetonitrile (37:63 v/v) as a mobile phase. The flow rate for optimum resolution and rapid separation was adjusted to 0.3 mL/min. Retention times of ursolic acid and uvaol were 3.05 and 3.74 minutes, respectively, as shown in Fig. 2. System suitability parameters are shown in Table 1.

Validation

Validation of the method was performed as per the ICH guidelines. (ICH, 2005)

Linearity in bulk powder

The developed method was shown to be reproducible in terms of peak shape and retention time. Under the chromatographic conditions described above, RSD% of ursolic acid and uvaol retention times in bulk powder were 0.9% (3.05 min ± 0.02) and 0.8% (3.74 min ± 0.03),

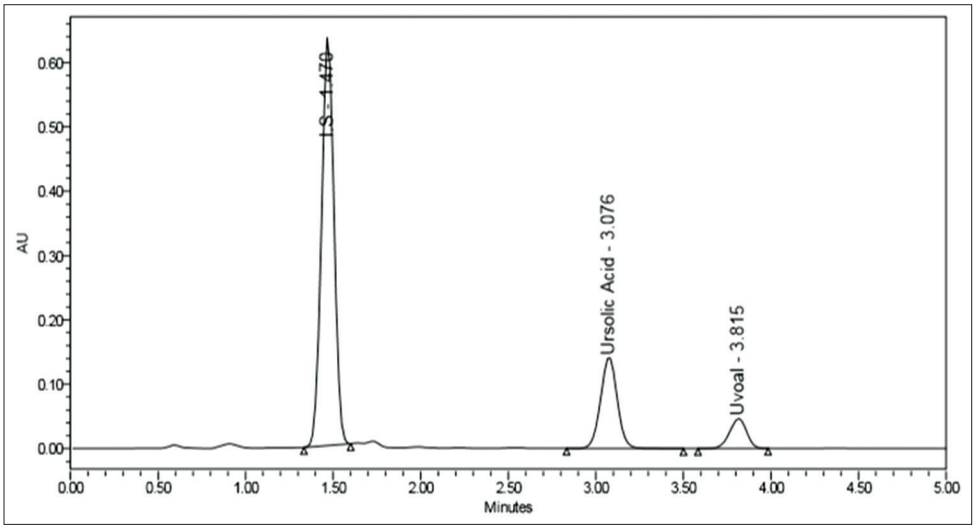

Fig 2. UPLC analysis of a sample containing 100 µg/mL of ursolic acid and uvaol as well as 20µg/ml of the internal standard

Table 2: Validation parameters of the proposed metho

Parameters	Ursolic acid		Uvaol	
	Samples in mobile phase	Samples after solid phase extracted	Samples in mobile phase	Samples after solid phase extracted
Range (µg/mL)	10–400	15–400	15–400	25–400
Regression Equation	y=0.00110x–0.0133	y=0.00104x–0.0141	y=0.00030x+0.002	y=0.00028x+0.001
Correlation coefficient (r)	0.9994	0.9996	0.9994	0.9998
LOQ (µg/mL)	10	15	15	25
LOD (µg/mL)	3.3	5	5	8.3
Intra-assay precision[a]	1.1	2.1	1.8	2.3
Inter-assay precision[a]	1.9	3.0	2.2	2.7
Recovery from dry olive leaves[b]	-	55.10 µg.g-1±0.72	-	314.29 µg.g-1±0.97
Recovery from dry oregano planted in Palestine[b]	-	25.57 µg.g-1±0.35	-	19.07 µg.g-1±0.38
Recovery from dry oregano planted in Spain[b]	-	28.70 µg.g-1±0.36	-	48.10 µg.g-1±0.62

a: expressed as the relative standard deviation (RSD). b: expressed as [mean of three samples taken from natural matrices]

respectively. Moreover, the peak areas of ursolic acid and uvaol concentrations were linear in the range of 10 - 400 µg/mL for ursolic and 15 - 400 µg/mL for uvaol with correlation coefficients (r) ≥ 0.999,a s shown Fig. 3. As the calibration curve performed in triplicates, the slopes and correlation coefficients showed high consistency which demonstrated the reliability of the standard curve over the concentration ranges studied as shown in Table 2.

Linearity after extraction by solid-phase extraction

The developed method was also applied to different concentrations of ursolic acid and uvaol extracted from the mobile phase using the extraction method described previously. Under the chromatographic conditions described above, the peak areas of extracted ursolic acid and uvaol concentrations were linear in the range of 15- 400 µg/mL for ursolic acid and 25-400 µg/mL for uvaol with correlation coefficients (r) ≥ 0.997. As the calibration curve performed in triplicates, the slopes and correlation coefficients showed high consistency which demonstrated the reliability of the standard curve over the concentration ranges studied as shown in Table 2.

Accuracy and precision

Data for intra- and inter- assay precision and accuracy were derived by the analysis of ursolic acid and uvaol in the mobile phase and after solid-phase extraction from the mobile phase samples in a single day (intra-) and within 10 days (inter-). As shown in Tables 3 to 6, the intra-assay RSD% values in mobile phase ranged from 0.5% to 1.1% for ursolic acid and from 0.9% to 1.8% for uvaol, whereas the inter-assay RSDs were 0.6% to 1.9% for ursolic acid and 1.0% to 2.2% for uvaol. The intra-accuracy ranges for ursolic and uvaol were 99.42 to 100.10% and 97.41% to 100.60%, respectively, while the inter-accuracy ranges were 96.30% to 97.74% for ursolic acid and 96.05% to 99.29% for uvaol. Moreover, the intra-assay RSDs% values for ursolic acid and uvaol in extracted by solid-phase extraction from the mobile phase ranged from 0.9% to 2.1% for ursolic acid and from 1.0% to 2.3% for uvaol, whereas the inter-assay RSDs% were 1.3% to 3.0% for ursolic acid and 1.2% to 2.7% for uvaol. The intra-accuracy for ursolic acid and uvaol after solid-phase extraction from the mobile phase were the range of 91.52% to 94.92% and 90.75% to 95.68%, respectively, while the inter-accuracy ranges were 89.68% to 92.55% for ursolic acid and 86.75% to 94.45% for uvaol. In the data collected, ursolic acid and uvaol were stable for at least 12 hours at room temperature and for at least 10 days, when stored at 4 °C for the combination in the mobile phase.

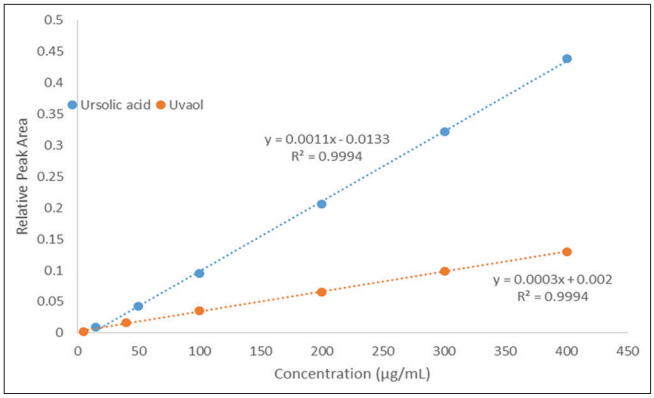

Fig 3. Calibration curve for ursolic acid and uvaol

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOQ for ursolic acid was 10 µg/mL whereas the LOQ for uvaol was 15 µg/mL. These concentrations gave a RSDs% of 1.1% and 1.8% for ursolic acid and uvaol, respectively. However, the LOD for ursolic acid was found to be 3.3 µg/mL and 5 µg/mL for uvaol using 10 µL as an injection volume. In contrast, the LOQ for ursolic acid and uvaol in the solid-phase extracted samples were found to be 15 µg/mL for ursolic acid and 25 µg/mL for uvaol. These concentrations gave an RSDs% of 2.1% and 2.3% for ursolic acid and uvaol, respectively. However, the LOD for ursolic acid and uvaol in the solid-phase extracted samples were found to be 5.0 µg/mL and 8.3 µg/mL, respectively, using 10 µL as an injection volume as shown in Table 2.

Extraction method used for analysis of ursolic acid and uvaol

As shown in Table 7, the extraction method was able to extract 95.49% of ursolic acid and 97.89% of uvaol from mobile phase. Ursolic acid and uvaol showed to have good stability and there weren't any degradation products detected. The amount of ursolic acid and uvaol extracted from the mobile phase by the used extraction procedure mentioned above were calculated from the calibration curve equation and was found to be equal to 190.98 µg/mL out

Table 3: Intra-assay precision and accuracy data for Ursolic acid and Uvaol determination in bulk powder using UPLC-UV

Ursolic acid µg/mL	Mean± SD (n = 3) observed/ µg/ mL	Precision[a] (%)	Accuracy[b] (%)	Uvaol µg/mL	Mean± SD (n = 3) observed/ µg/ mL	Precision[a] (%)	Accuracy[b] (%)
15	14.94±0.157	1.1	99.60	5.0	5.03±0.092	1.8	100.60
200	200.14±1.449	0.7	100.1	200	199.30±1.810	0.9	99.65
400	397.67±2.044	0.5	99.42	400	389.66±3.320	0.9	97.41

[a] expressed as the RSD. [b] expressed as [mean % deviation = mean calculated concentration/ nominal concentration X100]

Table 4: Inter-assay precision and accuracy data for Ursolic acid and Uvaol determination in bulk powder using UPLC-UV.

Ursolic acid concentration µg/mL	Mean± SD (n = 3) observed/ µg/ mL	Precision[a] (%)	Accuracy[b] (%)	Uvaol concentration µg/ mL	Mean± SD (n = 3) observed/ µg/ mL	Precision[a] (%)	Accuracy[b] (%)
15	14.86±0.284	1.9	96.30	5	4.92±0.107	2.2	98.40
200	198.96±1.886	0.9	97.74	200	198.57± 2.570	1.3	99.29
400	396.93±2.453	0.6	97.54	400	384.18±3.831	1.0	96.05

[a] expressed as the RSD. [b] expressed as [mean % deviation = mean calculated concentration/ nominal concentration X100]

Table 5: Intra-assay precision and accuracy data for Ursolic acid and Uvaol determination after solid phase extraction using UPLC-UV

Ursolic acid µg/mL	Mean± SD (n = 3) observed/ µg/ mL	Precision[a] (%)	Accuracy[b] (%)	Uvaol µg/mL	Mean± SD (n = 3) observed/ µg/ mL	Precision[a] (%)	Accuracy[b] (%)
25	22.88±0.483	2.1	91.52	20	18.15±0.417	2.3	90.75
200	185.10±1.998	1.1	92.55	200	186.11±2.539	1.4	93.05
400	379.69±3.430	0.9	94.92	400	382.72±3.863	1.0	95.68

Table 6: Inter-assay precision and accuracy data for Ursolic acid and Uvaol determination in bulk powder using UPLC-UV

Ursolic acid concentration µg/mL	Mean± SD (n = 3) observed/ µg/ mL	Precision[a] (%)	Accuracy[b] (%)	Uvaol concentration µg/mL	Mean± SD (n = 3) observed/ µg/ mL	Precision[a] (%)	Accuracy[b] (%)
25	22.42±0.672	3.0	89.68	20	17.35±0.472	2.7	86.75
200	181.41±2.959	1.6	90.70	200	183.57± 2.882	1.6	91.79
400	370.20±4.738	1.3	92.55	400	377.82±4.671	1.2	94.45

Table 7: Extraction recovery of ursolic acid and uvaol from mobile phase in triplicate using UPLC-UV

Ursolic acid (concentration range 50-400 µg.mL^{-1})	Extracted	Non-extracted	*Recovery (%)	Uvaol (concentration range 40- 400 µg.mL^{-1})	Extracted	Non-extracted	*Recovery (%)
1	0.00104	0.00110	94.54 %	1	0.00028	0.00030	93.33 %
2	0.00144	0.00151	95.63 %	2	0.00094	0.00100	94.00 %
3	0.00174	0.00182	95.60 %	3	0.00063	0.00060	91.30 %
Mean	0.00141	0.00148	95.49 %	Mean	0.00062	0.00063	97.89 %

*Recovery (%) = slope of the standard curve of extracted ursolic acid and uvaol / slopes of the standard curve of non-extracted ursolic acid and uvaol x 100

of 200 µg/mL of ursolic acid (95.49% of ursolic acid was recovered from the initial concentration of ursolic acid) and 195.78 µg/mL out of 200 µg/mL of uvaol (97.89% of uvaol was recovered from the initial concentration of uvaol).

Application of the method in natural matrices (olive leaves and oregano)

Ursolic acid and uvaol (Fig. 1) are both secondary metabolites of significant biological activity (Novotny et al., 2001; Wozniak et al., 2015; Mancha-Ramirez and Slaga, 2016; Ikeda et al., 2008; Giuffre, 2012). The properties of ursolic acid are very well explored compared to uvaol due to the higher stability of carboxylic functional group compared to the stability of the primary alcoholic group of uvaol that is easily prone to oxidation. Due to the lower stability of uvaol, its properties were investigated on a much smaller scale compared to ursolic acid. On the other hand, the presence of uvaol in dry plant material may suggest that the handling and manipulating of the plant material was reasonably gentle and at lower temperatures. The presence of both compounds in natural matrices is of interest to pharmaceutical and medical scientists, as well as to food processing. Consequently, the application of the fast and reliable method for ursolic acid and uvaol detection and quantification is of high interest. Therefore, we believe our UPLC method will have a great impact on ursolic acid and uvaol analysis in dry material of plant origin.

The separation of both substances and the internal standard was fast and excellent under the selected experimental conditions. The developed method was aimed to analyze two types of dry plant material, namely dry olive leaves (*Olea europaea L.*) and two brands of commercially available oregano which were cultivated in two different geographic areas; Palestine and Spain (*Origanum vulgare L*). These two matrices were selected due to the proven presence of ursolic acid and uvaol in these matrices as documented in the scientific literature (Olmo-Garcia et al., 2016; Fernandez-Hernandez et al., 2015; Guinda et al., 2010; Allouche et al., 2009). Moreover, ursolic acid and uvaol levels were noted to be relatively higher in olive tree parts than other natural

Table 8: Determined concentrations of ursolic acid and uvaol in of dry olive leaves and oregano

Matrix	*Ursolic acid*			*Uvaol*		
	Sample 1	**Sample 2**	**Sample 3**	**Sample 1**	**Sample 2**	**Sample 3**
Oregano (Al-Baraka)	25.65 µg/g	25.19 µg/g	25.88 µg/g	18.89 µg/g	19.50 µg/g	18.83 µg/g
Average±SD (standard deviation)		25.57 µg/g ± 0.35			19.07 µg/g±0.38	
*Precision (%)		1.4			2.0	
Matrix	Ursolic acid			Uvaol		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
Oregano (Waitro's)	28.67 µg/g	29.06 µg/g	28.35 µg/g	47.42 µg/g	48.66 µg/g	48.12 µg/g
Average±SD (standard deviation)		28.70 µg/g ± 0.36			48.10 µg/g±0.62	
*Precision (%)		1.3			1.3	
Matrix	Ursolic acid			Uvaol		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
Dry olives leaves	54.98 µg/g	55.88 µg/g	54.44 µg/g	314.45 µg/g	315.17 µg/g	313.26 µg/g
Average±SD (standard deviation)		55.10 µg/g ± 0.72			314.29 µg/g±0.97	
*Precision (%)		1.3			0.3	

***Expressed as the relative standard deviation (RSD)**

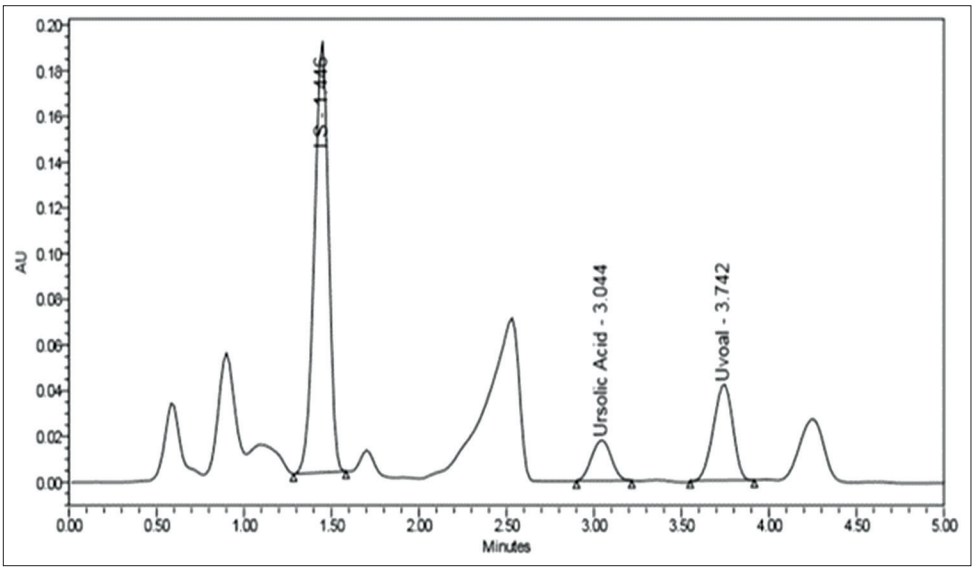

Fig 4. Determination of ursolic acid and uvaol in dried olive leaves in the presence of internal standard.

resources (Olmo-Garcia et al., 2016; Fernandez-Hernandez et al., 2015; Guinda et al., 2010). Additionally, triterpenes were found to be of higher concentrations (almost 30-times) in olive leaves than in the olive fruit, where they a located exclusively in the epicarp (21). A detailed analysis of forty olive cultivars of *Olea europaea* L. from the World Olive Germplasm Bank Collection of Cordoba (Spain) reported uvaol concentration ranging from 1.50 to 19.35 mg/kg (Allouche et al., 2009). The same team reported the triterpenoic acid concentration oscillating between 8.90 to 112.36 mg/kg while ursolic acid was detected only at the trace level concentrations (Allouche et al., 2009). The chromatogram of the extract of dry olive leaves obtained in our laboratory is presented at Fig. 4. Our data are in agreement with these findings (Allouche et al., 2009), as the concentration of ursolic acid determined by our team was lower than the concentration of uvaol (Table 8, 55.10 µg/g ± 0.72 for ursolic acid and 314.29 µg/g ± 0.97 for uvaol in olives dry leaves).

On the other hand, while the presence of ursolic acid in oregano was documented in oregano (Baranauskaite et al., 2016; Sowa et al., 2014; Nowak et al., 2013; Venkateswara Rao et al., 2011), the information on the presence of uvaol in oregano is only sporadic and its presence in plants was reported qualitatively but not quantitatively (Jager et al., 2009). Therefore, this study was designed to determine uvaol and ursolic acid concentrations in oregano cultivated in Palestine and Spain. The results of the performed analysis are presented in Fig. 5 and 6. The concentration of ursolic acid was lower in both samples of dry oregano compared to dry olive leaves (Table. 8). Moreover, the concentration of uvaol was significantly higher than ursolic acid in dry oregano cultivated in Spain

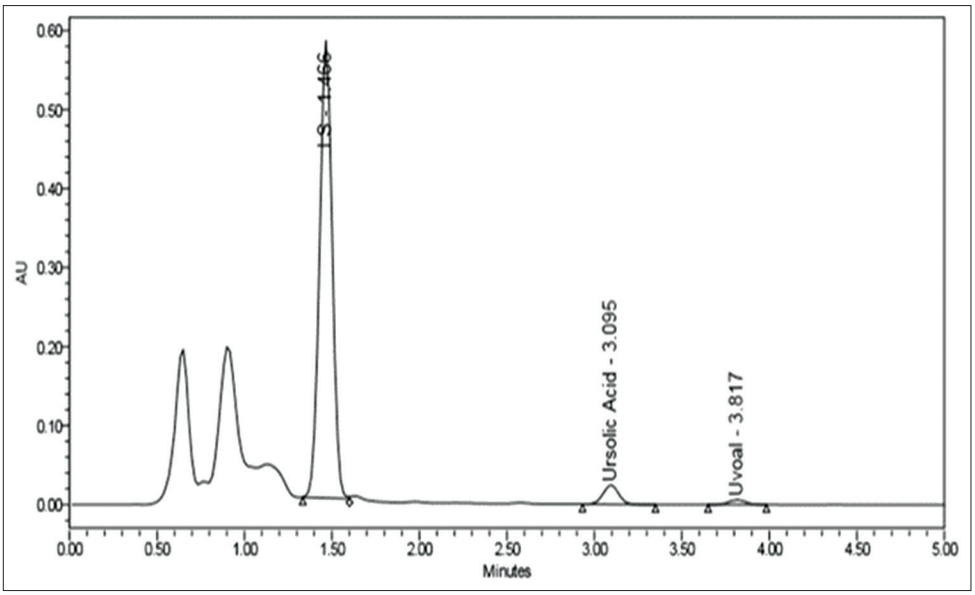

Fig 5. Determination of ursolic acid and uvaol in dry oregano (planted in Palestine) in the presence of internal standard.

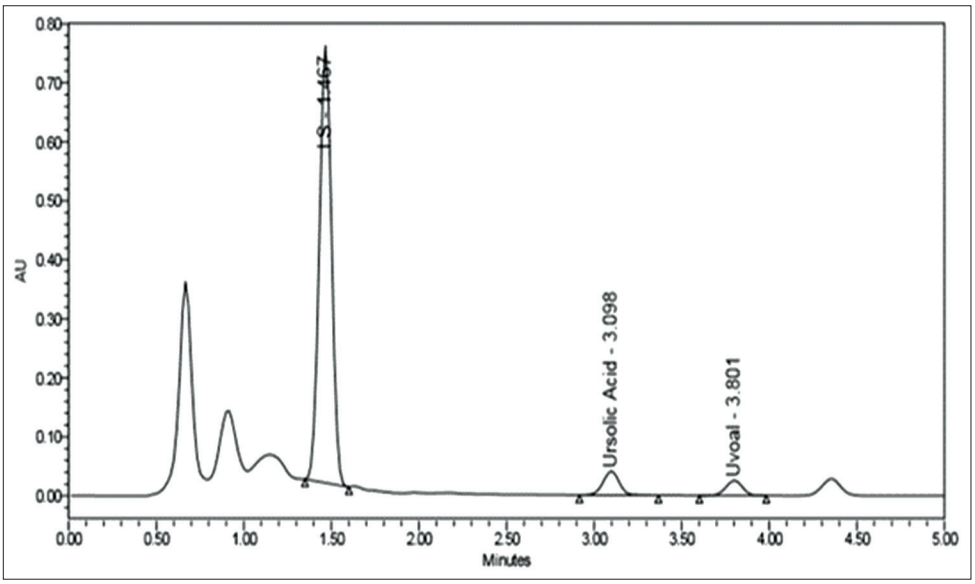

Fig 6. Determination of ursolic acid and uvaol in dry oregano (planted in Spain) in the presence of internal standard.

(Fig. 6, Table 8). However, small traces of uvaol in dry oregano planted in Palestine were detected by an in-depth analysis of the data from the chromatogram Fig. 5 by mass spectrometry application in all performed analyses. The obtained data are correlated with results already published (Jager et al., 2009).

The presence of uvaol and ursolic acid in the analyzed extracts was confirmed by the use of mass spectrometry. Mass spectrometry spectra of both uvaol and ursolic acid are presented in Fig. 7.

The obtained data are important, especially it shows that the UPLC method used was able to determine the presence of relatively unstable uvaol in plant material. The levels of uvaol found might be affected by the different origins (including climatic conditions) of the sample or, in the case of the same origin, it may signal the 'age' of the sample or storage under suboptimal condition leading to uvaol oxidation to ursolic acid. Additionally, it needs to stated that concentrations of secondary metabolite in samples of natural origin are affected by many aspects (i.g. by georaphical area of production of plants, microclimate, agronomic conditions, used fertlizers, irrigation, by specific cultivar, degree of ripening etc.). Our method may help in elucidating effects of this various factors on ursolic acid and uvaol concentrations in plants.

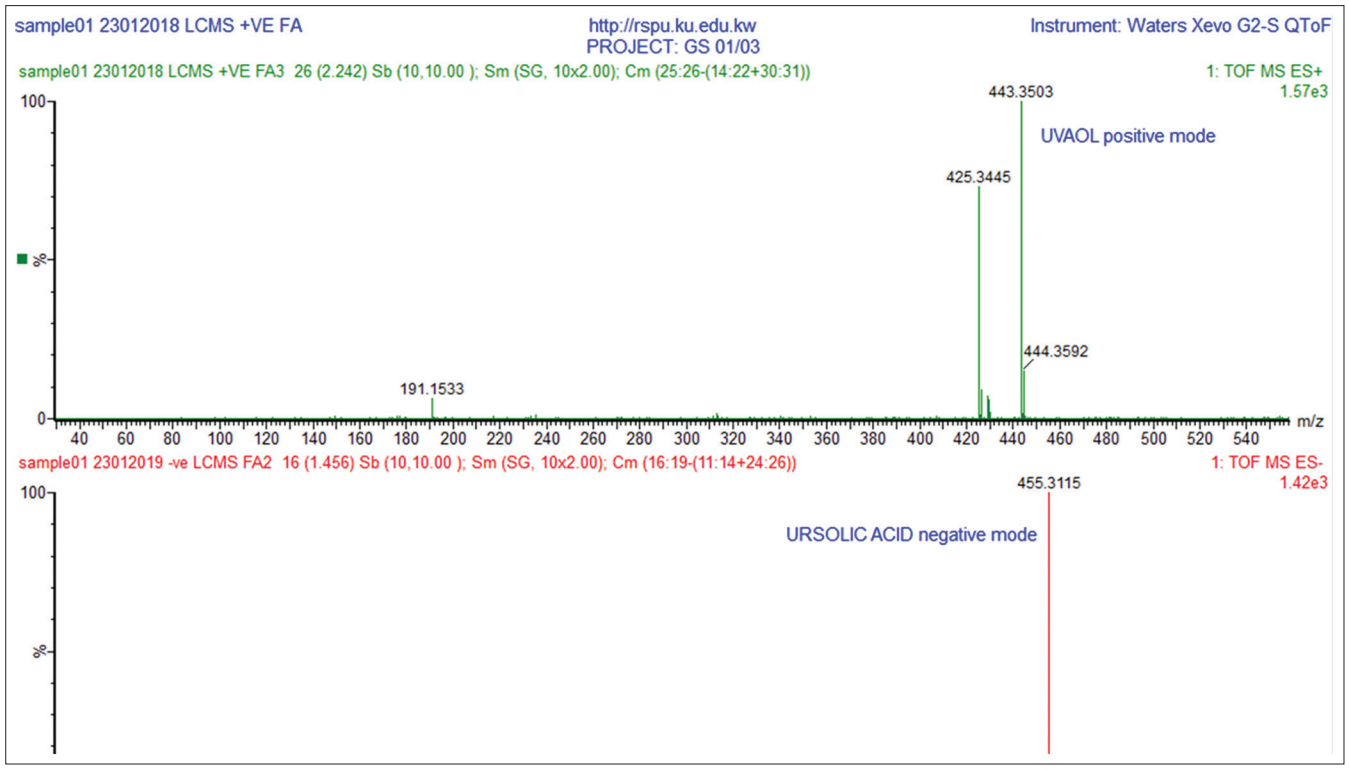

Fig 7. MS Spectra of ursolic acid (negative mode) and uvaol (positive mode) performed on mass spectrometry.

There are some interesting published data dealing with applications of instrumental analytical methods in determination of ursolic acid. For example, LC-MS (SIM mode) was used in determining concentrations of three triterpenoid acids, ursolic acid was included, in various oregano species and chemotypes (28). The authors achieved excellent linearity in calibration curves but confirmed that "significant variation in chemical composition between species and within a species was found" (Shen et al., 2010). In our study, we were able to determine ursolic acid and uvaol qualitatively and quantitatively in natural matrices and shown that UPLC-MS method is suitable to determine our analytes of interest in samples with different ratios. Moreover, the use of UPLC method is less time consuming than the use of LC methods.

CONCLUSION

The presented data indicate the suitability of UPLC methods for the fast determination of natural origin compounds that are present in natural matrices in different concentrations. Determination of more sensitive secondary metabolites, uvaol in our case, may indicate the method suitability for quality control purposes. The developed method is fast, sensitive and reliable as well as it can be applied in qualitative and quantitative analysis of ursolic acid and uvaol in various matrices of plant origin.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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