Full paper/Mémoire

Nettle (*Urtica dioica* L.) as a source of antioxidant and anti-aging phytochemicals for cosmetic applications

L'ortie (Urtica dioica L.), une source de produits antioxidants et phytochimiques anti-âge pour des applications en cosmétique

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ABSTRACT

Nettle (*Urtica dioica* L.) is a herbaceous perennial that has been used for centuries in folk medicine. More recently, nettle extracts have also been used in cosmetics because of the many benefits of their topical application for skin health. Their potential anti-aging action is of particular interest and is primarily ascribed to their antioxidant capacity. Here, using an experimental design approach and a clustering analysis, we linked the phytochemical composition of nettle extracts to their biological activities. This approach confirmed the antioxidant capacity of nettle extracts as well as providing the first evidence of another mechanism for their anti-aging potential involving the inhibition of enzyme activities, such as elastase and collagenase. We attributed these inhibitory effects to ursolic acid and quercetin present in the nettle extracts. Our results also demonstrated the possibility of extracting ursolic acid, quercetin and other phenolic compounds differentially to obtain an extract with a strong antioxidant capacity and anti-aging activities toward both elastase and collagenase. This could be of particular interest for cosmetic applications of nettle extracts.
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R É S U M É

L'ortie (*Urtica dioica* L.) est une plante herbacée vivace utilisée depuis des siècles en médecine traditionnelle. Plus récemment, les extraits d'ortie ont été également utilisés dans des produits cosmétiques en raison des nombreux effets bénéfiques de leurs applications topiques sur la santé de la peau. En particulier, leur potentielle action anti-âge présente un fort intérêt et a été essentiellement attribuée à leur fort pouvoir antioxydant. Dans cette étude, en utilisant un plan d'expérience et une analyse hiérarchisée, nous avons connecté la composition phytochimique d'extraits d'ortie à leurs activités biologiques. Cette approche nous a permis de confirmer la capacité antioxydante des extraits d'ortie, mais aussi de proposer la première preuve d'un autre mécanisme de leur potentiel anti-âge impliquant l'inhibition d'activités enzymatiques. En effet, nos travaux montrent que ce second mécanisme implique l'inhibition d'enzymes de dégradation de la matrice extracellulaire telles que l'élastase et de la collagénase, et ces effets inhibiteurs ont été attribuables principalement à l'acide ursolique et la quercétine présents dans les extraits d'ortie. Nos résultats ont également mis en évidence la possibilité d'extraire de manière différentielle l'acide ursolique, la quercétine et d'autres composés phénoliques pour obtenir un extrait avec une forte capacité antioxydante et une activité anti-âge au travers de l'inhibition à la fois de l'élastase et de la collagénase, ce qui pourrait être d'un intérêt particulier pour les applications cosmétiques des extraits d'ortie.

1. Introduction

Nettle (*Urtica dioica* L.) is a widespread wild plant that is also cultivated for specific uses. This herbaceous perennial belonging to the *Urticaceae* family is very common in temperate climate regions. Its genus name is derived from the Latin *urere*, which means to sting, and more precisely from *uro*, meaning to burn by friction. Its bright, dark green and serrated leaves as well as its stem are covered with erect glandular hairs containing formic acid and histamine, which are responsible for these unpleasant effects. This plant has been used for centuries in folk medicine to cure a wide range of diseases or disorders such as arthritis, rheumatism and eczema [1]. A more recent use of polar extracts from nettle roots is the treatment of benign prostatic hyperplasia [2] while its foliar extracts display potent anti-inflammatory action [3]. Nettle extracts are also used in cosmetics and their topical application presents many benefits for skin health (anti-inflammatory and anti-swelling, for example). Nettle is also a common component of shampoo. However, one of the most interesting actions of nettle extracts for cosmetic applications results from their antioxidant and anti-aging properties. Nettle accumulates a number of reactive oxygen species scavengers, which can reduce free radical damage to the skin and therefore has anti-aging effects [4–6]. To date, the molecule(s) responsible for this action and its rationale are largely unknown. Besides the antioxidant action, anti-aging activity could also be due to the inhibition of degrading enzymes, such as elastase or collagenase, whose actions result in a loss of skin elasticity leading to wrinkles [7]. Among the molecules present in nettle extracts, ursolic acid and quercetin [5,6] are the most interesting for the development of anti-aging action. Ursolic acid, which mainly accumulated in nettle roots, is a well-known elastase inhibitor [8] whereas quercetin, which mainly accumulated in the leaves, is one of the most prominent antioxidants [9]. However, the antioxidant action of quercetin is not simple, and its pro-oxidant activity in certain circumstances is widely documented in the literature [10,11]. The pro-oxidant state of quercetin may provide insights into its apparent in vitro mutagenicity [12]. Because of these side effects, quercetin is no longer used in cosmetics. Here, a factorial design approach was used to produce extracts with contrasting concentrations of ursolic acid and quercetin. Their corresponding antioxidant and anti-aging (anti-collagenase and anti-elastase) actions were evaluated. Coupled with a hierarchical clustering analysis, this approach enabled a role to be ascribed to ursolic acid and quercetin and a mechanism for the anti-aging action of nettle extracts to be proposed.

2. Materials and methods

2.1. Plant material

Urtica dioica L. seeds were obtained from Nova-Flore and the plantlets resulting from seedlings were cultivated in a phytotronic room at 25 °C under a 16-h photoperiod (30 µmol m^{-2} s^{-1} photosynthetically active radiation). Three-month-old whole plants were collected and lyophilized prior to extraction.

2.2. Chemicals and reagents

All extraction solvents (water, ethanol, and acetonitrile) used in the present study were of analytical grade and were obtained from Thermo Scientific. Ursolic acid and quercetin standards were from Sigma-Aldrich. All reagents for the determination of total phenolic compounds, antioxidant activity, and collagenase and elastase activity were from Sigma-Aldrich.

2.3. Extraction procedure

The sample (500 mg of whole plant lyophilized powder) was suspended in 20 mL of an extraction solvent and homogenized using a blender (Ultraturrax, T25 basic) for 1 min at 19,000 rpm. Addition of 1 M of hydrochloric acid was used to hydrolyze the glycosidic bonds in order to simplify chromatogram analysis. Extraction duration, temperature and ethanol concentration were selected as variables to obtain nettle extracts with contrasting concentrations of ursolic acid, quercetin and other phenolic compounds. These 3 independent variables were coded at 3 levels (Table 1) and 27 batches were prepared using a 3^3 full factorial experimental approach (Table 2).

2.4. HPLC and LC–MS analysis

Ursolic acid and quercetin were determined using a Varian HPLC system equipped with an online degasser (Metachem Degasit), an autosampler (Prostar 410) and a photodiode array detector (PDA, Prostar 335). The separation was performed at 35 °C on an RP-18 column (250 × 4.0 mm id, 5 μm; Purospher Merck). The mobile phase was composed of acetonitrile (solvent A) and 0.1% (v/v) formic acid acidified ultrapure water (solvent B). The composition of the mobile phase varied during a 1-hour run according to a linear gradient ranging from a 5:95 (v/v) to 100:0 (v/v) mixture of solvents A and B, respectively, at a flow rate of 0.6 ml/min. Detection was performed at 210 and 254 nm. Ursolic acid and quercetin were identified by comparison with authentic standards, standard additions and LC–MS analysis performed on a Water 2695 Alliance coupled with a single quadrupole mass spectrometer ZQ. LC-ESI-MS data were collected in the positive and negative modes. Data acquisition and processing were performed with MassLynx 4.0 software. Ursolic acid and quercetin were quantified against 5-point calibration curves (Table 3).

2.5. Chromatographic validation

The HPLC separation was validated for intraday and interday precision, repeatability and accuracy. The linear correlations between peak area and standard concentrations were found to be high, in the range of 50 to 1,000 ng/mL for DPT. The resulting linear equation and R^2-value for a 5-point calibration graph was >0.999, and the slope of five replicates of the calibration graph covering the analytical range for the ursolic acid and quercetin standards varied no more than 1% in terms of RSD over a period of four weeks. The LOD (signal/noise = 3) and LOQ (signal/noise = 10) were also determined. The RSDs of retention times during the validation procedure were satisfactory (less than 0.65%). The precision was evaluated by five injections of the same sample on the same day (intraday variation) and on 5 consecutive days (interday variation). Repeatability was evaluated by applying the whole extraction procedure three times to the same batch of material. The recovery was determined by standard addition at 4 chosen levels (0 ng, 10 ng, 50 ng and 100 ng added to aliquots of the same extract) and the resulting percentage recovery was determined following the whole extraction process.

2.6. Determination of the total content of phenolic compounds

The total phenolic content was determined using the Folin-Ciocalteu (Sigma) reagent as described in [13]. All measurements were carried out in triplicate. Gallic acid (Sigma) was the standard for the calibration curve, and the total phenolic contents were expressed as mg gallic acid equivalents per 100 g of DW.

2.7. Antioxidant activities

The antioxidant capacity of each nettle extract was determined by the ferric reducing/antioxidant power (FRAP) and the cupric reducing antioxidant capacity (CUPRAC) methods as described in [14] and [15] respectively. All measurements were performed in triplicate and the antioxidant capacity of each extract was expressed in Trolox C equivalent antioxidant capacity (TEAC) measured with a standard 1 mM concentration of Trolox C.

2.8. Collagenase assay

Clostridium histolyticum collagenase (Sigma) was used for this assay and collagenase activity was estimated spectrophotometrically using *N*-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA; Sigma) as the substrate and following the decrease in absorbance of FALGPA at 335 nm for 20 min. Each measurement was carried out in triplicate and the assay was performed according to the protocol described in [7]. The anti-collagenase activity was expressed as an inhibition percentage relative to the corresponding control (addition of the same volume of the extraction solvent) for each extract or as the IC_{50} value for ursolic acid and quercetin standards using ED50plus v1.0 software.

2.9. Elastase assay

Porcine pancreatic elastase (Sigma) was used for this assay and the elastase activity was estimated

Table 1
Validation parameters of the HPLC method developed for quantification of ursolic acid and quercetin in nettle extracts.

	Regression equation	R^2	Linear range	LOD (ng)	LOQ (ng)	Precision (%RSD)		Repeatability (%RSD)	Recovery (%)
						Intraday	Interday		
Ursolic acid	$Y = 0.213x \pm 0.1107$	0.998	50–1000	1.15	3.84	0.48	0.94	3.51	103.7
Quercetin	$y = 0.1126x \pm 2.1546$	0.999	50–1000	0.42	1.38	1.24	2.64	2.70	94.3

Values are the mean and RSDs of 5 replicates.

Table 2
Coded levels and experimental values of the 3 independent variables.

Independent variables	Code units	Coded variable levels		
		−1	0	+1
Time (min)	X_1	30	90	120
Temperature (°C)	X_2	25	45	65
EtOH (% v/v)	X_3	50	75	100

spectrophotometrically using *N*-Succ-Ala-Ala-Ala-*p*-nitro-anilide (AAAVPN; Sigma) as the substrate and following the release of *p*-nitroaniline at 410 nm. Each measurement was carried out in triplicate and the assay was performed according to the protocol described in [7]. The anti-elastase activity was expressed as percentage inhibition relative to the corresponding control (addition of the same volume of extraction solvent) for each extract or as the IC_{50} value for

Table 3
Measured experimental values determined for individual design points.

Batch #	Extraction conditions [a]			Phytochemicals [b]			Anti-aging activities [c]		Antioxidant activities [d]	
	X_1	X_2	X_3	Ursolic acid	Quercetin	Phenolics	Collagenase	Elastase	CUPRAC	FRAP
1	-1	-1	-1	5.00	35.58	86.90	16.23	12.75	0.58	0.27
2	-1	-1	0	3.76	33.67	367.74	14.97	11.38	1.95	0.53
3	-1	-1	1	0.28	2.30	278.10	1.03	0.80	1.37	0.57
4	-1	0	-1	4.13	28.06	283.14	12.87	10.19	1.40	0.47
5	-1	0	0	3.81	27.23	410.07	12.42	9.75	2.17	0.60
6	-1	0	1	1.98	7.83	317.96	3.92	3.42	1.57	0.60
7	-1	1	-1	14.38	17.64	217.74	12.81	14.65	1.16	0.31
8	-1	1	0	14.02	11.27	372.55	10.12	12.74	1.98	0.54
9	-1	1	1	2.93	12.68	362.04	6.24	5.35	1.85	0.69
10	0	-1	-1	3.19	22.20	225.77	10.15	8.00	1.20	0.38
11	0	-1	0	2.43	29.42	464.45	12.74	9.35	2.22	0.58
12	0	-1	1	0.99	3.57	265.40	1.83	1.62	1.33	0.55
13	0	0	-1	5.21	30.55	215.77	14.30	11.59	0.77	0.27
14	0	0	0	4.47	25.26	482.34	11.89	9.70	2.71	0.57
15	0	0	1	1.53	7.06	304.23	3.43	2.90	1.57	0.61
16	0	1	-1	25.40	2.87	162.92	11.31	18.53	0.61	0.18
17	0	1	0	27.74	11.20	428.66	15.57	22.33	2.37	0.43
18	0	1	1	3.80	11.97	409.85	6.31	5.77	1.96	0.70
19	1	-1	-1	3.15	21.85	116.93	10.00	7.88	0.53	0.19
20	1	-1	0	3.25	27.81	456.57	12.42	9.50	2.45	0.44
21	1	-1	1	2.32	5.81	287.88	3.25	3.13	1.28	0.57
22	1	0	-1	7.79	21.87	178.18	11.87	11.14	0.70	0.22
23	1	0	0	3.56	15.44	427.88	7.60	6.51	2.26	0.63
24	1	0	1	1.82	10.98	290.80	5.12	4.13	1.41	0.61
25	1	1	-1	20.34	0.47	127.52	8.32	14.36	0.56	0.15
26	1	1	0	32.41	6.99	396.57	15.76	24.51	2.16	0.41
27	1	1	1	5.55	10.33	356.93	6.35	6.57	1.69	0.73

[a] Extraction conditions: see Table 1 for values; [b] Phytochemical concentrations are given in µg/g DW except for phenolic compound content given in mg/g of gallic acid equivalent; [c] Anti-aging activities are given in % of inhibition relative to the control (extraction solvent); [d] Antioxidant activities are given in TEAC (Trolox C equivalent antioxidant capacity). Values presented are means of 3 independent replicates.

ursolic acid and quercetin standards using ED50plus v1.0 software.

2.10. Statistical analysis

For the 3^3 full factorial experimental approach, the experiments were carried out in triplicate and the response at each design point was recorded. Data were then subjected to regression analysis using least-square regression methodology to obtain the parameters of the mathematical model. Student's *t*-test was used to check the statistical significance of the regression coefficients derived from the model. Analysis of variance (ANOVA) was then applied to evaluate the statistical significance of the proposed model. Surface plots of the response as a function of the independent variables were obtained using the fitted model. All data are expressed as the mean and the standard deviation of at least 3 independent experiments. Comparative statistical analysis of groups was performed using ANOVA performed by the DOE (design of experiments) analysis of the XL-STAT software. Hierarchical clustering analysis was performed using MeV 4.4 software using the Pearson correlation and following log2 transformation of the data.

3. Results and discussion

3.1. HPLC method validation

The presence of ursolic acid in nettle root extracts and quercetin in leaf extracts has been described previously [5,6,16]. An HPLC method was developed to determine ursolic acid (Fig. 1B) and quercetin (Fig. 1B) simultaneously in nettle extracts (Fig. 1A). The separation conditions used were adapted from a UPLC method developed to quantify quercetin and betulinic acid, a more hydrophilic derivative of ursolic acid, in *Disporopsis pernyi* extracts [17]. Ursolic acid and quercetin were separated on a C18-grafted reverse phase column using an HPLC linear gradient composed of a mixture of acetonitrile and 0.1% (v/v) formic acid acidified water, ranging from 5% to 100% acetonitrile during a one-hour run at a flow rate of 0.6 ml per minute. A typical HPLC chromatogram (with detection set at 254 nm) of a nettle whole plant extract showing the separation of these compounds is presented in Fig. 1C. Addition of standards as well as LC–MS analysis (m/z 455 [M−H]$^−$ for ursolic acid and m/z 301 [M−H]$^−$ for quercetin) confirmed their respective identities. Here, besides the confirmation of the presence of these molecules in nettle, our results also evidenced the possibility of their simultaneous extraction from the whole plant.

To ensure precision and accuracy, this separation method was validated and the results are presented in Table 1. A good linearity of the calibration curves of the peak areas (y) against the injected quantities (x) over the analyzed range (50–1000 ng) was observed for ursolic acid (210 nm) and quercetin (254 nm). The limits of detection (LOD) and quantification (LOQ) were as low as 1.15 ng and 3.84 ng for ursolic acid, and 0.42 ng and 1.38 ng for quercetin, respectively. The HPLC method was suitable in terms of both intraday and interday precision and

repeatability as indicated by the small variations measured for the corresponding relative standard deviations (RSDs), ranging from 0.48% to 2.64% for the intraday and interday variations and less than 3.51% for repeatability (Table 1). These low variations confirmed the adequate precision of the method developed. The recovery rates, using 3 levels of chosen additions, of between 94.3% for quercetin and 103.7% for ursolic acid indicated the good accuracy of the method. Taken together, these results demonstrated that this HPLC procedure is well suited to the simultaneous determination of ursolic acid and quercetin contents in nettle extracts.

3.2. Generation of nettle extracts with contrasting concentrations of ursolic acid, quercetin and phenolic acids

The second objective of this study was to develop a versatile and fast extraction system to generate batches of whole plant extracts from nettle with very contrasting ursolic acid and quercetin contents. For this purpose, a factorial experimental design approach was used to generate these extracts with a simple method, maceration. It is well established that extraction efficiency can be influenced by multiple parameters such as the duration, temperature or polarity of the solvent. These three parameters were evaluated logically: extraction time (coded as X_1, with experimental values ranging from 30 to 90 min), extraction temperature (coded as X_2, with experimental values ranging from 25 to 65 °C) and ethanol percentage (X_3, with experimental values ranging from 50 to 100% (v/v) in a mixture with water). The coded and experimental values of these 3 independent variables are presented in Table 2. The choice of solvent is generally based on the polarity of the target molecule to be extracted and that of undesirable compounds, its extraction efficiency and also cost and safety requirements [18]. In our work, ethanol mixed with water was chosen as the extraction solvent for its low toxicity and ease of removal under reduced pressure. It is also a classic solvent for the extraction of natural compounds with a wide range of polarity; for example, addition of water in ethanol is known to be a simple way to increase the extraction of more polar compounds [18]. Following the determination of the experimental values of the ursolic acid, quercetin and phenolic compound contents, multiple regression led to the corresponding second order polynomial equation corresponding to the extraction yield as a function of the 3 independent variables studied (Supplementary Table 1). The quality of the fit of the 3 predictive models was satisfactory as indicated by the coefficient of determination values ranging from 0.817 to 0.884 and *p*-values always lower than 0.0001 for the ANOVA (Supplementary Tables 1 and 2). According to the values measured and the response surface plots, ethanol concentration appeared to be the most critical parameter for the extraction of each compound (Table 3, Fig. 2). High EtOH concentration significantly favored the extraction yield of phenolic compounds whereas it resulted in a significant decrease in the extraction yield of both quercetin and ursolic acid (Supplementary Table 1). The extraction temperature also appeared to affect the extraction yields

A

B

quercetin
(PubChem CID 5280343)

ursolic acid
(PubChem CID 64945)

C
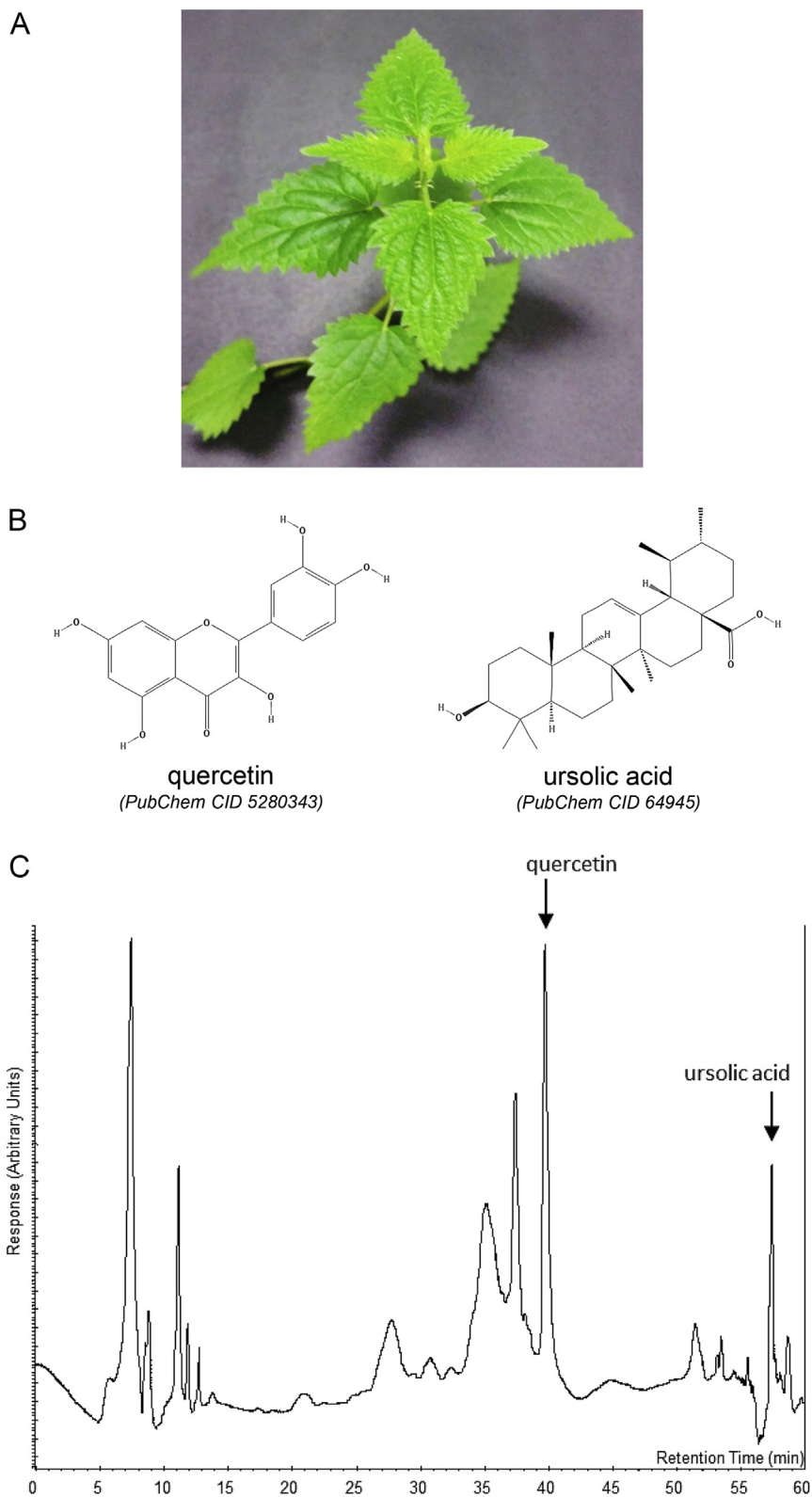
Fig. 1. A. Three-month old nettle plant growing under greenhouse conditions. B. Ursolic acid and quercetin structures. C. Typical chromatogram of a nettle extract showing the presence of ursolic acid and quercetin (detection at 254 nm).
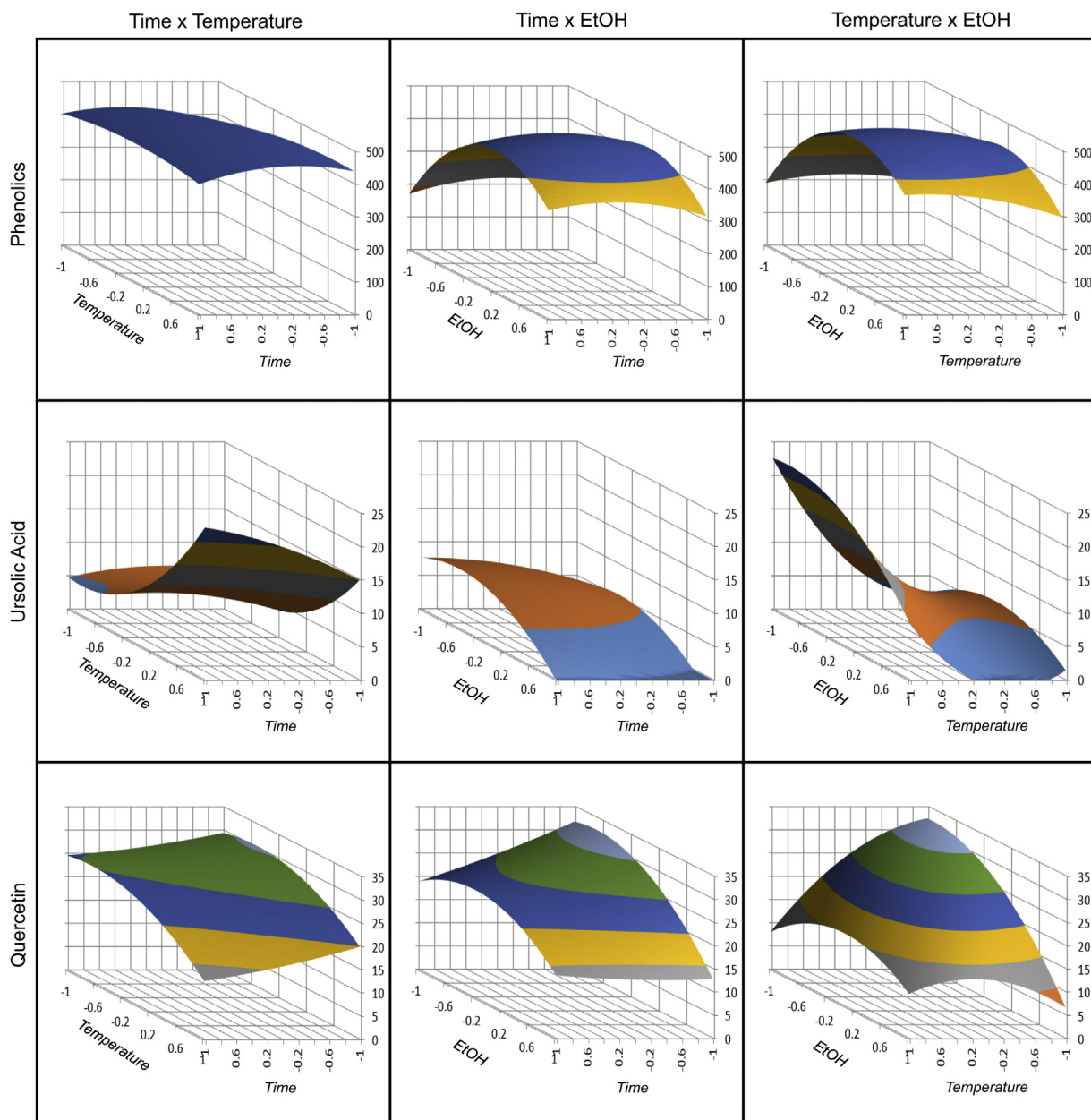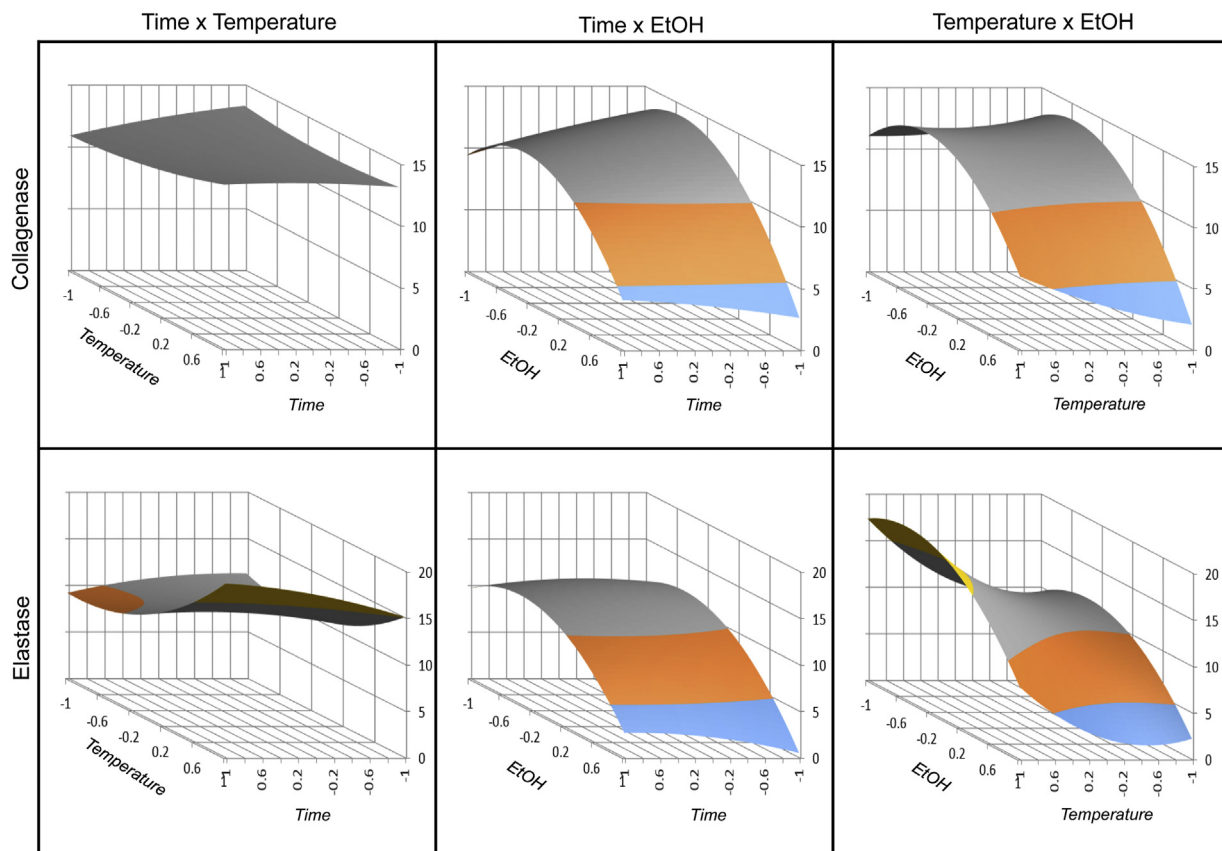
Fig. 2. Response surface plots for the effects of extraction time, temperature and ethanol concentration on ursolic acid, quercetin and total phenolic compound contents in nettle extracts.

significantly: high extraction temperatures increased the ursolic acid content but hindered quercetin extraction (Supplementary Table 1). In our hands, the best yields were obtained following a 90-min extraction with 75% (v/v) EtOH at 65 °C for ursolic acid, a 30-min extraction with 50% (v/v) EtOH at 25 °C for quercetin and a 45-min extraction with 75% (v/v) EtOH at 45 °C for phenolic compounds. The batches resulting from the experimental design presented contrasting concentrations of ursolic acid (from 0.28 to 32.41 mg per 100 g DW), quercetin (from 0.47 to 35.58 mg per 100 g DW) and total phenolic

compounds (from 86.90 to 482.34 mg per 100 g DW of gallic acid equivalent).

3.3. Antioxidant and anti-aging activities of nettle extracts

The next stage consisted of i) evaluating the antioxidant and anti-aging activities of these extracts and ii) constructing predictive models for these activities (Fig. 3 and 4) in order to establish correlations between these biological activities and the phytochemical composition of the nettle

Fig. 3. Response surface plots for the effects of extraction time, temperature and ethanol concentration on the antioxidant capacity of the nettle extracts determined using the FRAP and CUPRAC assays.

extracts, particularly their contents in ursolic acid, quercetin and phenolic acids (Fig. 5).

3.3.1. Antioxidant capacities

The antioxidant capacity of the extracts given by the CUPRAC and FRAP assays revealed a strong antioxidant activity of some batches ranging from 0.53 (batch 19) to 2.71 (batch 14) Trolox C equivalent antioxidant capacity (TEAC) using the CUPRAC assay and from 0.15 to 0.73 TEAC using the FRAP assay (Table 3).

The difference observed in the TEAC determined by FRAP and CUPRAC assays could be explained by the physicochemical nature of the antioxidant evidenced by these two tests. The FRAP method is used to evaluate the antioxidant activity of hydrophilic substances whereas CUPRAC is applied to both lipophilic and hydrophilic antioxidants [19]. Here, our results indicated that the main contributors to this antioxidant activity were primarily hydrophilic substances.

Considering that the present extracts constituted a complex mixture of anti- and pro-oxidant compounds, compared to the standard Trolox C, the measured antioxidant capacities revealed the presence of at least one strong antioxidant molecule in the nettle extracts. The predictive models for the antioxidant capacity of nettle extracts were statistically significant for both FRAP and CUPRAC assays

(Supplementary Tables 1 and 2). The resulting response surface plots revealed a strong correlation between the antioxidant activities and the phenolic acid concentrations of the extracts (Fig. 3, Supplementary Table 1). The hierarchical clustering analysis (HCA) confirmed this observation and indicated that quercetin was not the main contributor to the antioxidant activity of nettle extracts (Fig. 5). The antioxidant action of quercetin is not simple, and its pro-oxidant activity in certain circumstances is widely documented in the literature [10,11]. The presence of other phenolic compounds in the nettle extracts may be important to stabilize the antioxidant action of quercetin. This observation appeared in good agreement with published results indicating the presence of other potential antioxidant compounds such as flavonoids (kaempferol derivatives, for example) or lignans (secoisolariciresinol, for example) in nettle extracts [6,16].

3.3.2. Anti-aging activities

The anti-aging action of these extracts was evaluated by their ability to inhibit elastase and collagenase activities because these enzymes cause the degradation of the extracellular matrix proteins in the dermis, resulting in skin alterations such as deep wrinkles, loss of skin tonus and resilience [7]. The inhibitory effect of each extract on
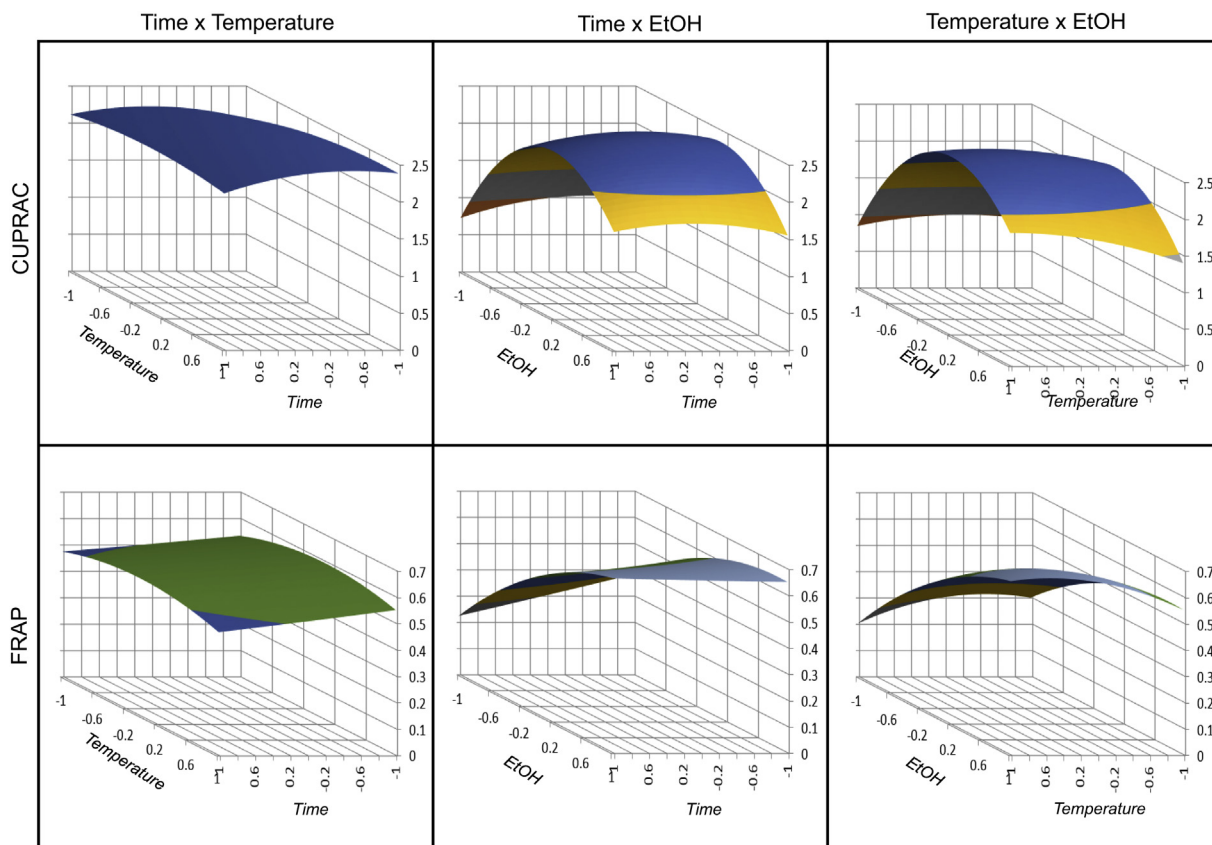
Fig. 4. Response surface plots for the effects of extraction time, temperature and ethanol concentration on the anti-aging activity of the nettle extracts based on their inhibitory action on elastase and collagenase activities.
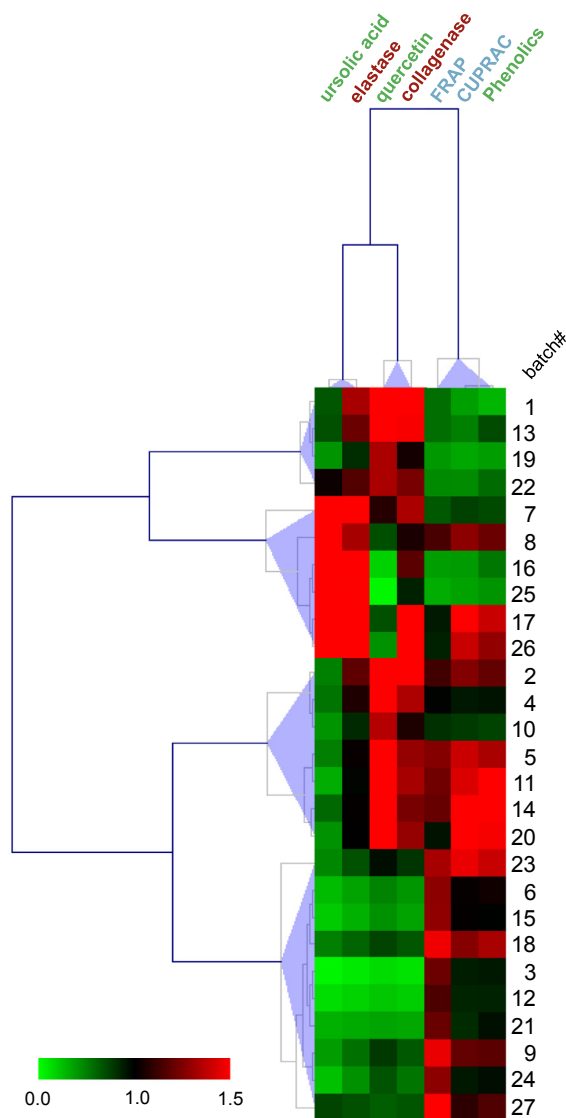
collagenase and elastase activity is presented in Table 3. The inhibitory potentials were up to 16.23% for collagenase inhibition (batch 1, Table 3) and up to 24.51% for elastase inhibition (batch 26, Table 3). The deduced anti-aging models predicting the inhibitory effect of each extract on collagenase and elastase activities were confident and statistically significant (Supplementary Tables 1 and 2). Interestingly, these models indicated that the less polar substances (better extracted with pure EtOH) were not responsible for these inhibitory effects, as confirmed by the response surface plots (Fig. 4). As for ursolic acid extraction, a positive impact of higher extraction temperature was also noted for elastase inhibitory action (Fig. 4, Supplementary Table 1). A strong positive correlation between elastase inhibition and the ursolic acid content of the extract was observed and confirmed by the HCA (Fig. 5). In the same way, a positive correlation between collagenase inhibition and the quercetin content of the corresponding nettle extracts was observed (Fig. 5). Nevertheless, we also noted that the presence of ursolic acid alone could be important for collagenase inhibition since some batches containing a small amount of quercetin but rich in ursolic acid (7, 8, 16, 25, 17, 26) displayed a strong inhibition of collagenase (Fig. 5, Table 3). Similar inhibitions of elastase by ursolic acid and collagenase by quercetin have been described [8,20]. Nevertheless, to the best of our knowledge, the

present work constitutes the first report on the potential anti-aging action of nettle extracts resulting from the inhibition of these extracellular matrix-degrading enzymes.

Interestingly, it was possible to obtain extracts with both antioxidant and inhibitory properties toward elastase and collagenase (batches 17 and 26; Table 3, Fig. 5) with 75% (v/v) EtOH at an extraction temperature of 65 °C; these extracts were rich in ursolic acid and total phenolic compounds.

3.3.3. Comparison with commercial standards

Finally, the antioxidant and anti-aging potential of nettle extracts was compared to those of commercial standards of ursolic acid and quercetin. These assays confirmed that quercetin, one of the most prominent antioxidants [9], displayed a much better antioxidant capacity than ursolic acid. However, they also showed that the nettle extracts exhibited a very attractive scavenging activity (Table 4). Our HCA evidenced that quercetin was certainly not the most prominent antioxidant present in nettle extracts but might act with other phenolic compounds (Fig. 5). Considering the oxidant duality of quercetin [10,11], these other phenolic compounds may favor its antioxidant action. The effects of ursolic acid and quercetin on elastase and collagenase activities were then estimated (Table 4). Ursolic acid was the most effective against elastase activity with an IC_{50}

Fig. 5. Hierarchical clustering analysis of the nettle extracts as a function of their phytochemical composition, antioxidant capacity and inhibitory action on elastase and collagenase activities.

of 75.3 μM whereas quercetin was the most effective against collagenase activity with an IC$_{50}$ of 79.3 μM, confirming our HCA results. These results with standards were in good agreement with our data obtained with nettle extracts when considering that batch 1, which displayed a collagenase inhibition of 16.23%, contained c. 29 μM of quercetin while batch 26, which displayed an elastase inhibition of 24.51%, reached a concentration of c. 35 μM of ursolic acid. Interestingly, in the in vitro tests with commercial standards, these two compounds were relatively efficient at inhibiting both collagenase and elastase (Table 4). Additional experiments (data not shown) revealed a mixed inhibition mode for ursolic acid against elastase whereas all the other inhibitory actions described in the present work were found non-competitive. Evidence of a binding site for ursolic acid on elastase, responsible for its inhibitory action, was described in [8]. Several substrates can be used to measure elastase activity and competitive or non-competitive inhibition was observed depending on the substrate. This discrepancy was explained by the occurrence of 5 independent active sub-sites in the elastase enzyme, binding to which depends on the substrate [8]. Quercetin was found to be the most active natural flavonoid among those tested for the in vitro inhibition of collagenase [20].

4. Conclusions

The antioxidant activity of nettle (*Urtica dioica* L.) extracts has been described previously and, to date, this has been suggested as being responsible for the anti-aging activities of nettle. Here, we confirm this observation but also provide the first evidence of an additional mechanism involving the inhibition of collagenase and elastase activities. This could be ascribed to ursolic acid and quercetin present in the nettle extracts. Considering the potential side effects of quercetin, our results also demonstrated the possibility of choosing extraction parameters to enable an efficient differential extraction of ursolic acid, quercetin and other phenolic compounds in order to obtain extracts with a very low quercetin content. Here, we showed that such extracts display a strong antioxidant capacity and potential anti-aging activities due to their inhibitory effects on elastase and collagenase, which could be of particular interest for cosmetic applications of nettle extracts.

Appendix A. Supplementary data

Supplementary data related to this article can be found in the online version, at http://dx.doi.org/10.1016/j.crci.2016.03.019.

Table 4
Antioxidant and anti-aging activities of commercial standards of ursolic acid and quercetin.

	Anti-aging		Antioxidant	
	Elastase[a]	Collagenase[a]	CUPRAC[b]	FRAP[b]
Urtica dioica extract	nd	nd	1.56 ± 0.1	0.47 ± 0.03
Ursolic acid	75.3 ± 1.0	97.5 ± 0.3	0.49 ± 0.01	0.14 ± 0.03
Quercetin	107.6 ± 0.3	79.3 ± 0.1	4.32 ± 0.3	2.51 ± 0.2

nd: not determined.
[a] IC$_{50}$ given in μM.
[b] In TEAC (Trolox C equivalent antioxidant activity), this value is relative to the antioxidant activity given by Trolox C (1 mM).

References

[1] R. Upton, J. Herb. Med. 3 (2013) 9–38, http://dx.doi.org/10.1016/j.hermed.2012.11.001.

[2] M. Schöttner, D. Ganßer, G. Spiteller, Planta Med. 63 (1997) 529–532, http://dx.doi.org/10.1055/s-2006-957756.

[3] B. Obertreis, K. Giller, T. Teucher, B. Behnke, H. Schmitz, Arznei-mittelforschung 46 (1996) 52–56.

[4] C.A. Rice-Evans, N.J. Miller, G. Paganga, Free Radic. Biol. Med. 20 (1996) 933–956, http://dx.doi.org/10.1016/0891-5849(95)02227-9.

[5] S. Shailajan, H. Harshada, S. Dipti, T. Bhavesh, J. Appl. Pharm. Sci. 4 (2014) 92–95.

[6] B. Otles, S. Yalcin, Sci. World J. (2012) 1–12, http://dx.doi.org/10.1100/2012/564367.

[7] J. Wittenauer, S. Mäckle, D. Sußmann, U. Schweiggert-Weisz, R. Carle, Fitoterapia 101 (2015) 179–187, http://dx.doi.org/10.1016/j.fitote.2015.01.005.

[8] Q.L. Ying, A.R. Rinehart, S.R. Simon, J.C. Cheronis, Biochem. J. 277 (1991) 521–526. http://www.biochemj.org/content/277/2/521.abstract.

[9] A.W. Boots, L.C. Wilms, E.L.R. Swennen, J.C.S. Kleinjans, A. Bast, G.R.M.M. Haenen, Nutrition 24 (2008) 703–710, http://dx.doi.org/10.1016/j.nut.2008.03.023.

[10] E.J. Choi, K.-M. Chee, B.H. Lee, Eur. J. Pharmacol. 482 (2003) 281–285, http://dx.doi.org/10.1016/j.ejphar.2003.09.067.

[11] S. Fiorucci, J. Golebiowski, D. Cabrol-Bass, S. Antonczak, Proteins Struct. Funct. Bioinforma 67 (2007) 961–970, http://dx.doi.org/10.1002/prot.21380.

[12] M. Harwood, B. Danielewska-Nikiel, J.F. Borzelleca, G.W. Flamm, G.M. Williams, T.C. Lines, Food Chem. Toxicol. 45 (2007) 2179–2205, http://dx.doi.org/10.1016/j.fct.2007.05.015.

[13] I. Dini, G.C. Tenore, A. Dini, Food Chem. 113 (2009) 411–419, http://dx.doi.org/10.1016/j.foodchem.2008.07.053.

[14] D. Komes, A. Belščak-Cvitanović, D. Horžić, G. Rusak, S. Likić, M. Berendika, Phytochem. Anal. 22 (2011) 172–180, http://dx.doi.org/10.1002/pca.1264.

[15] L. Rimkiene, L. Ivanauskas, A. Kubiliene, K. Vitkevicius, G. Kiliuviene, V. Jakstas, J. Anal. Methods Chem. (2015) 1–7, http://dx.doi.org/10.1155/2015/280167.

[16] D. Orčić, M. Francišković, K. Bekvalac, E. Svirčev, I. Beara, M. Lesjak, et al., Food Chem. 143 (2014) 48–53, http://dx.doi.org/10.1016/j.foodchem.2013.07.097.

[17] Y. Wang, S. Li, D. Han, K. Meng, M. Wang, C. Zhao, J. Anal. Methods Chem. 2015 (2015), http://dx.doi.org/10.1155/2015/130873.

[18] L. Yu, S. Haley, J. Perret, M. Harris, Food Chem. 78 (2002) 457–461, http://dx.doi.org/10.1016/S0308-8146(02)00156-5.

[19] A. Ratz-Lyko, J. Arct, K. Pytkowska, Ski. Res. Technol 18 (2012) 421–430, http://dx.doi.org/10.1111/j.1600-0846.2011.00588.x.

[20] B.Y. Sin, H.P. Kim, Arch. Pharm. Res. 28 (n.d.) 1152–1155. http://dx.doi.org/10.1007/BF02972978.