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Extraction and quantification of luteolin and apigenin from artichoke (*Cynara cardunculus* var. *scolymus* (L.) cv. Madrigal) by-products

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RESUMEN

Los tallos, brácteas exteriores, y hojas al ser subproductos del procesamiento de las alcachofas representan una gran preocupación para las industrias, debido a que se acumulan en toneladas semanales y por lo tanto ejercen un impacto considerable en el medio ambiente. El presente estudio investiga el uso de estos subproductos industriales provenientes de *Cynara cardunculus* var. *scolymus* (L.) cv. Madrigal como una fuente de flavonas que pueden ser usadas como nutracéuticos. Estas flavonas fueron extraídas siguiendo cuatro protocolos diferentes que incluyeron: (A) material seco y molido extraído con etanol, (B) una Extracción Asistida por Ultrasonido (EAU) de material seco y molido macerado con etanol, (C) material seco homogenizado con etanol, y (D) material fresco homogenizado con etanol. Se utilizó un equipo HPLC y se llevó a cabo una separación isocrática con detección UV para cuantificar la cantidad de flavonas obtenidas mediante cada protocolo de extracción. Se encontró que el cultivar Madrigal rindió más apigenina que luteolina. En cuanto a la flavona apigenina, los mejores métodos de extracción fueron A (37 mg/kg peso seco) y B (61 mg/kg peso seco); el mejor método de extracción de luteolina fue el que usó EAU, el cual mostró un rendimiento de sólo 9 µg/kg en peso seco.

ABSTRACT

Stems, outer bracts, and leaves represent a huge concern for industries as by-products from the processing of artichokes, accumulating in tonnes per week and thus exerting considerable impact on the environment. The present study investigated the use of these industrial by-products from *Cynara cardunculus* var. *scolymus* (L.) cv. Madrigal as a source of flavones that might be used as neutraceuticals. These flavones were extracted according four different protocols that included (A) dried and ground material extracted with ethanol, (B) Ultrasound-Assisted Extraction (UAE) of dried and ground material extracted with ethanol, (C) dried material homogenized with ethanol, and (D) fresh material homogenized with ethanol. An HPLC isocratic separation with UV-detection was used to quantify the amounts of flavones obtained with each extraction protocol. It was found that the cultivar Madrigal yielded more apigenin than luteolin. For the former, the best extraction protocols were A (37 mg/kg dry weight) and B (61 mg/kg dry weight); for the latter, the best extraction protocol, that using UAE, yielded just 9 µg/kg dry weight.

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INTRODUCTION

Artichoke (*Cynara cardunculus* L.) is an herbaceous plant native to the Mediterranean Basin which has a well-recognized history of consumption as food and various uses in traditional medicine. The edible part of the plant is the enlarged receptacle and the tender, thickened bases of the bracts of the head (*capitulum*), which is the immature inflorescence used worldwide as both a fresh and canned delicacy (Lombardo *et al*., 2010a). In addition, artichoke leaf extracts have been documented since ancient times as a traditional folk medicine mainly for its choleretic, diuretic and hypocholesterolemic effects (Fritsche *et al*., 2002).

Today, production of artichoke is widely diffused all over the world with an average of 130 000 ha being cultivated in 2011. Europe is the leading producer with approximately 749 000 metric tonnes per year, followed by the Americas with 339 000 metric tonnes per year, then finally Africa and Asia with about 305 000 and 154 000 metric tonnes per year, respectively (FAO, 2013). Industrial by-products from artichoke processing (a heterogeneous mixture of stems, outer bracts, and leaves) represent about 80% of the biomass (Ceccarelli *et al*., 2010). These by-products accumulate in tones per week thus exerting significant environmental impacts and a potential money loss for many companies. These materials have been used alternatively as a raw material for animal feed, sources of organic mass, fuel, and for fiber production (Sanchez-Rabaneda *et al*., 2003).

Amongst the various polyphenols in the diet, luteolin and apigenin correspond to flavones, are the most abundant components of the flavonoids, and have been identified in artichokes (Jaganath and Crozier, 2010). Many therapeutic effects have been attributed to these compounds, and have been shown to possess antimutagenic, antioxidative, antiallergic, antibacterial, antifungal, antiviral, and anti-inflammatory activities (Veličković *et al*., 2007). The concentration of these compounds in artichokes is affected by different factors, such as genotype, environmental conditions, crop management, and processing practices (Pandino *et al*., 2011; Pandino *et al*., 2012a).

The extraction of bioactive compounds can be performed on frozen, dried or fresh plant material. Solvent extraction using methanol and ethanol are most commonly applied when extracting plant materials due to their ease of use, efficiency, and wide applicability (Dai and

Mumper, 2010). It is important to consider that all the variables involved in the extraction, such as temperature and time, influence the recovery of phenolic compounds (Robards, 2003). Chew *et al*. (2011) reported that temperatures up to 65 ^{o}C could enhance the recovery of phenolic compounds whereas temperatures above 65^{o}C can cause a rapid compound degradation. Ultrasound is a technology that can be used to improve extraction efficiency, since it helps in the disruption of biological membranes thus facilitating the release of extractable compounds and enhances the penetration of solvent into cellular materials improving mass transfer (Dai and Mumper, 2010).

The objective of this research was to investigate the efficiencies of four different protocols on the extraction of luteolin and apigenin from the industrial by-products of processing "Madrigal", a previously unstudied cultivar of artichoke.

EXPERIMENTAL

Plant Material

Industrial by-products of the artichoke cultivar Madrigal (a heterogeneous mixture of stems, outer bracts, and leaves) were provided by PROCECONSA S.A. (Quito, Ecuador). They were collected on three separate occasions in February, May, and July of 2013. The by-products were thoroughly washed with potable tap water and stored at a temperature of -18 ^{o}C until analysis.

Chemicals and reagents

Acetonitrile, methanol (both HPLC grade) and o-phosphoric acid (85%) were obtained from Fisher Scientific (Pittsburgh, PA, USA); ethanol (96%) and acetic acid (100%) (analytical grade) were obtained from Merck (Darmstadt, Germany). HPLC analytical standards apigenin aglycone (99%) and luteolin aglycone (97%) were acquired from Sigma Aldrich (St Louis, MO, USA).

Extraction procedure

The extraction procedure was based on previous studies performed by Veličković *et al.* (2007). Plant material was either used fresh or oven dried at 50±5 ^{o}C. Four extraction protocols were applied. Protocol A consisted of ground dried plant material extracted with ethanol; protocol B was an ultrasound-assisted extraction (UAE) of ground dried plant material extracted with ethanol which was immersed in an ultrasonic bath (VWR model 751), operating at a temperature of 40 ^{o}C for 20 min and a frequency of 40 kHz; protocol C involved dried plant material homogenized with ethanol in a domestic blender; and protocol D consisted of fresh plant material homogenized with ethanol in a domestic blender. In all of the protocols 15 g of material were used and the ratio of plant material to extracting solvent was 1:10 m/V. The suspensions resulting from the four methods were stored at room temperature in the absence of light for 48 h. Afterwards, the liquid extract was separated from the plant debris by centrifuging three times for 8 min at 3000 *g*. The supernatant was recovered and

concentrated under reduced pressure at 59±1 ^{o}C, yielding a paste-like extract which was stored at -18 ^{o}C until analysis.

Sample preparation

Plant extracts (50±5 mg) were dissolved in 10 mL of methanol (HPLC grade). These were immersed in an ultrasonic bath to facilitate dissolution and then filtered through syringe filters (13 mm diameter, 0.45 µm pore size; Millipore Corp.) before being injected into the HPLC system.

Chromatographic conditions

The method for the HPLC analysis was based upon and adapted from previous research (Chen and Xiao, 2010). Chromatographic analyses were performed using a LC-10AD Liquid Chromatography System (Shimadzu) equipped with a RP-C_{16} column (4.6x250 mm, particle size 5 µm, 120 Å; Dionex) with a SPD-10AV UV-VS detector (Shimadzu). The column temperature was kept at 30 ^{o}C. The mobile phase consisted of methanol-acetonitrile-acetic acid-phosphoric acid-H_2O in a ratio of 200:100:10:10:200 V/V. It was vacuum-filtered and degassed in an ultrasonic bath. The sensitivity was set at 0.01 AUFS and the flow rate was 1.00 mL/min. The chromatograms were registered at 352 nm and the volume of sample injected was 20 µL. The software responsible for data processing was Chromeleon (v6.80, SR10, Build 2818; Activate Corp).

Identification and quantification of flavones

Standard calibration curves were generated for each flavone using 5 different concentrations which were: 0.007, 0.014, 0.021, 0.028 and 0.035 mg/mL for apigenin and 0.007, 0.014, 0.021, 0.028 and 0.035 µg/mL for luteolin. Calibration curves were fitted with linear regression. The plant samples were dissolved in methanol and analyzed in duplicate, being filtered as described above. Luteolin and apigenin were identified by their retention times and quantified according to the calibration curve.

Statistical analysis

Data were transformed with a logarithmic scale in order to fit a normal distribution. The results were subjected to an analysis of variance (ANOVA) with the consideration of a p-value of 0.05 as significant. Means were separated through the Tukey's Honestly Significant Difference (HSD) test. Analyses were carried out in SPSS Statistics (v.20, IBM).

RESULTS AND DISCUSSION

Apigenin and luteolin (Figure 1) showed retention times of 9.04 min and 6.96 min respectively (Figure 2).

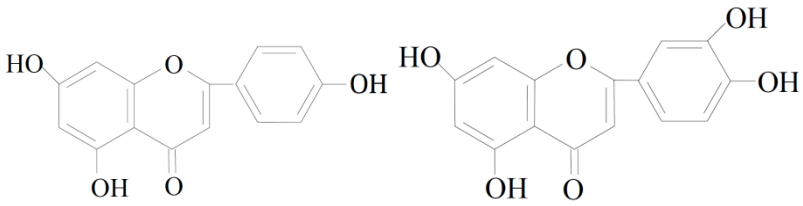

Figure 1. Structures of apigenin and luteolin (Veličković *et al.*, 2007)

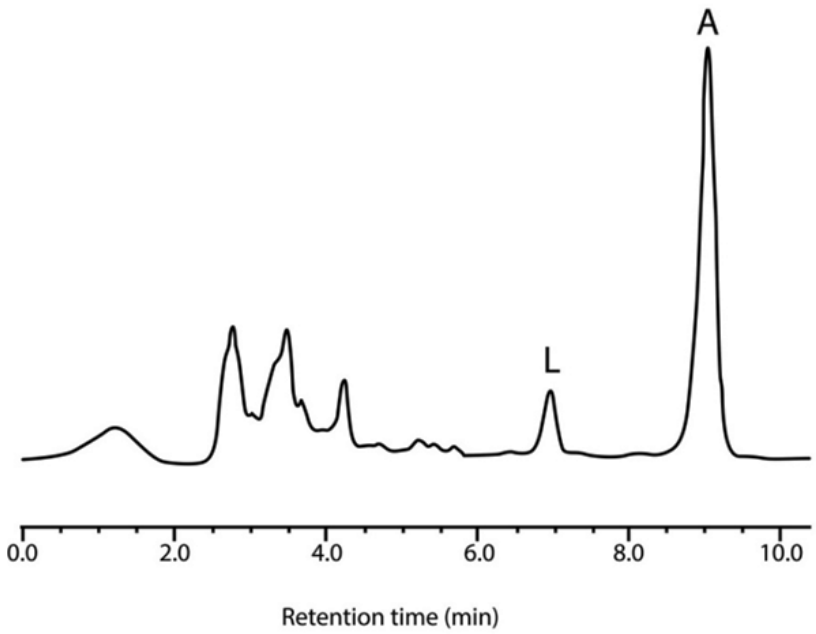

Figure 2.Separation of the flavones luteolin (L) and apigenin (A) with a detection wavelength of 352 nm. Ethanolic extract of dried and ground plant material. Chromatographic conditions are described in the Experimental Section.

The calibration curves for the analyzed flavones had excellent correlation coefficients (*r*), with *r*=0.9956 for luteolin and *r*=0.9925 for apigenin (Figure 3& 4). The repeatability of the HPLC analysis, measured by the Relative Standard Deviation (RSD) is 6.3% for apigenin and 4.6%

for luteolin, considered satisfactory since the majority of phytochemical studies present a range of 3-6% for RSD (Chen and Xiao, 2010).

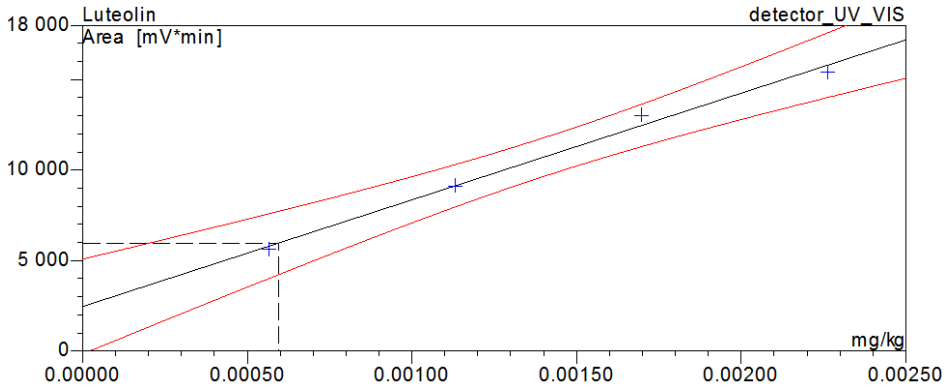

Figure 3. Calibration curve for luteolin.

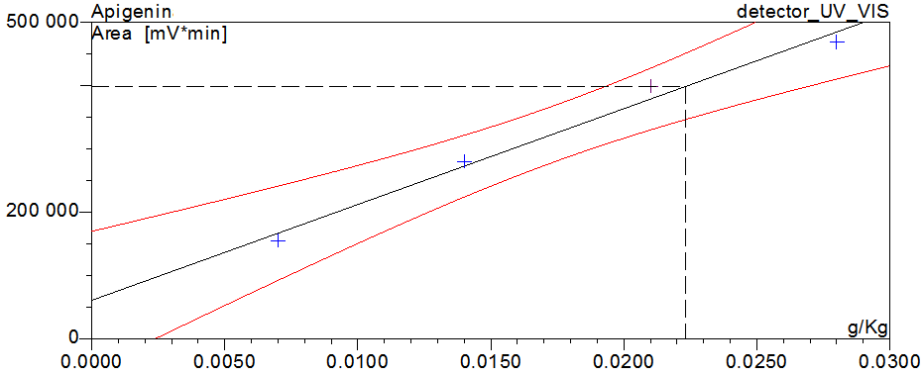

Figure 4. Calibration curve for apigenin.

The suitability of the system parameters (Table 1) including Resolution (R_s), Plate Number (N) and Asymmetric factor (A_s) of the HPLC system were established as adequate levels (Kaila *et al*., 2011).

Table 1.System suitability parameters for the analysis of apigenin and luteolin.

Parameter	Luteolin and Apigenin	Preferable levels[a]
R_s[b]	5.45	>3
A_s[c]	0.80	<2
N[d]	6933	>2000

[a](Kaila *et al*., 2011)
[b]Rs: Resolution
[c]As: Asymmetric factor
[d]N: Plate number

The different ethanolic extraction protocols yielded diverse results on the amount of flavones extracted (Table 2). Regarding apigenin, treatments A (dried and ground) and B (dried, ground and ultrasonic assisted) proved to be better among the four extraction protocols, whereas D (homogenized fresh material) extracted the least amount of flavones. On the other hand, treatments A, B and D were equally efficient for the extraction of luteolin from the plant material. When comparing the amount of flavones quantified with each other, apigenin content is greater than that of luteolin, consistent with the results of previous studies (Pandino *et al*., 2012a; Pandino *et al*., 2012b) which found substantial amounts of apigenin but only traces of luteolin in some clones of the Fiori cultivar. Relative high concentrations of apigenin constitute an interesting discovery given the fact that apigenin aglycones are rarely found in food plants (Justesen *et al*., 1998).

For both flavones, treatments that involved drying and grinding (A), and drying, grinding, and ultrasonic-assisted extraction (UAE) (B) were shown to be the most effective. This may be attributed to the higher extraction yields of phenolics from ground samples due to the improved extraction that occurs when particle size is smaller (Khoddami *et al*., 2013), and the positive effects of using UAE which has been shown to be an effective and promising technique for obtaining bioactive substances in less time (Veličković *et al*., 2006; Veličković *et al*., 2002) and in greater yield than when using maceration alone (Veličković *et al*., 2007).

Concentrations of luteolin were significantly lower than those of apigenin in extracts of this artichoke cultivar. However, an intriguing and inexplicable anomaly arises where the extraction efficiency of homogenizing fresh plant material was similar to that of extracting dried and ground plant material (A) or dried, ground and ultrasonically treated plant material (B), contrary to the trend shown for the extraction of apigenin (Table 2).

The time of sampling also influenced the extraction yield of flavones. Extraction efficiencies for both luteolin and apigenin were highest for plant samples taken in July (Table 2). Many factors affect the flavonoid content of a plant, such as area of cultivation, climatic and environmental conditions, vegetative phase (Georgieva *et al*., 2011), harvest time, geophysical conditions (Srivastava and Gupta, 2009), crop management, processing practices (Pandino *et al*., 2012b), post-harvest handling (Lattanzio *et al*., 1994), and exposure to light (Pinelli *et al*., 2007). Additionally, artichoke has a high oxidase potential

due to enzymes such as ascorbate oxidase, polyphenol oxidase, cytochrome oxidase and peroxidase (Gil *et al*., 1998), with polyphenol oxidase (PPO) responsible for most of the loss of flavone compounds (Espín and Wichers, 2000). Polyphenol oxidase activity can be activated by mechanical damage during post-harvest treatment (Lattanzio *et al*., 1994) and industrial processing. The higher extraction yield in July might be due to factors favoring flavone synthesis, while the lower extractions efficiencies in samples collected in February and May might be due to less synthesis and/or more degradation.

Table 2.Apigenin and luteolin concentrations in different extracts of the by-products of processing the artichoke cultivar Madrigal.

Treatment/Sampling date	Flavone (dry weight)		Flavone (fresh weight)	
	Apigenin (mg/kg)	Luteolin (µg/kg)	Apigenin (mg/kg)	Luteolin (µg/kg)
Dried and ground (A)				
February	28.82	0.99	0.86	0.15
May	33.82	2.09	1.72	0.31
July	48.97	13.65	0.81	2.05
Dried, ground, ultrasound (B)				
February	30.50	1.31	1.33	0.20
May	56.24	6.14	0.65	0.92
July	96.73	21.66	0.47	3.25
Dried and homogenized (C)				
February	16.11	0.74	1.56	0.11
May	23.61	0.48	1.82	0.47
July	13.25	0.70	2.53	0.11
Fresh and homogenized (D)				
February	0.07	4.62	0.01	0.69
May	0.01	7.51	0.00	1.11
July	0.01	7.83	0.00	1.16
Means				
Dried and ground (A)	37.20	5.58	1.13	0.84
Dried, ground, and ultrasound(B)	61.16	9.70	0.82	1.46
Dried and homogenized (C)	17.66	0.64	1.97	0.23
Fresh and homogenized (D)	0.03	6.65	0.00	0.99
Sampled in February	18.87	1.92	0.94	0.29
Sampled in May	28.42	4.05	1.05	0.70
Sampled in July	39.74	10.96	0.95	1.64

The mean concentrations of apigenin in the first three extracts, namely 32, 61 and 18 mg/kg dry weight (Table 2) were comparable to those reported by Pandino *et al.*(2012a) in the cultivar Spinosa di Palermo and its clones (Table 3). Concentrations of luteolin in the Madrigal, however, were considerably less, presenting concentrations in micrograms rather than milligrams.

Table 3: Apigenin and luteolin content (mg/kg of dry matter) of the outer bracts in relation to genotype (Pandino *et al*., 2012a)

Compound	Genotype						
	Spinoso di Palermo	Clone I	Clone II	Clone III	Clone IV	Clone V	Clone VI
Luteolin	17 ± 1	20 ± 1	12 ± 0.2	34 ± 3	52 ± 4	nd[a]	21 ± 2
Apigenin	43 ± 1	33 ± 2	37 ± 0.7	49 ± 1	160 ± 10	90 ± 7	99 ± 1

[a]nd = Not Detected

Other food sources are rich in the flavones apigenin and luteolin (Table 4) and the yields of apigenin extracted from the by-products of processing Madrigal are of a similar order, suggesting that these might represent an alternative dietary source once extracted and presented in the format of a food supplement.

Table 4: Apigenin and luteolin content in broccoli, bell pepper, onion leaves and celery (Miean and Mohamed, 2001)

Sample	Content, mg/kg dry matter	
	Luteolin	Apigenin
Broccoli	74.5 ± 0.05	ND[a]
Bell pepper	ND[a]	272.0 ± 0.02
Onionleaves	391.0 ± 0.05	ND[a]
Celery	80.5 ± 0.05	338.5 ± 0.04

[a]nd = NotDetected

CONCLUDING REMARKS

The results corroborate previous work that established the presence of the flavones apigenin and luteolin in artichoke in their aglycone form (Christaki *et al*., 2012; Farag *et al*., 2013; Lombardo *et al*., 2010b; Lopez-Lazaro, 2009; Sanchez-Rabaneda *et al*., 2003; Shimoda *et al*., 2003; Wang *et al*., 2003). This study, however, is the first to confirm their presence in the industrial by-products of processing artichoke. Unlike other commercial cultivars of artichoke, Madrigal showed very low concentrations of luteolin. The concentrations of apigenin that can be extracted, especially with a protocol that involves the use of ethanol and ultrasonic disruption, merits further investigation of an industrial scale extraction of the by-products of artichoke processing as a possible neutraceutical source of apigenin.

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