Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi
Iğdır University Journal of the Institute of Science and Technology

An LC-MS/MS Method Validation for the Phytochemical Quantification of Four Edible Plants

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ABSTRACT: A comprehensive LC-MS/MS method was developed and validated for the quantification of eight plant phytochemicals (cynarin, caffeic acid, syringic acid, *p*-coumaric acid, *o*-coumaric acid, vanillic acid, ferulic acid, chlorogenic acid) in plants. The developed analytical method was fully validated in terms of linearity, accuracy (recovery), inter and intra-day precision (repeatability), limits of detection and quantification (LOD/LOQ) and relative standard uncertainty (U% at 95% confidence level (k=2)). Chromatographic separation was performed on a reverse phased UHPLC. MS detection was performed using a triple quadrupole mass spectrometer and negative or positive ionization modes were optimized for each analyte. Multiple reaction monitoring (MRM) was used to quantify the analytes, related molecular ions and transition ions were optimized. After method validation, the phytochemical composition of methanolic extracts of some edible plants including artichoke (Cynara scolymus L.), broccoli (Brassica Oleracea var. Italica), cauliflower (Brassica Oleracea var. Botrytis) and tumble thistle (Gundelia Tournefortii) were investigated by the developed and validated LC-MS/MS method. Among the analysed plants, artichoke was by far the richest one in terms of phenolics. Additionally, chlorogenic acid was the most abundant phenolic compound in all plants. Although the studied edible plants were chosen as real samples, the developed LC-MS/MS method is applicable to a wide range of species in plant kingdom.

Keywords: Edible plants LC-MS/MS, method validation, phytochemicals.

Yenilebilir Dört Bitki Türünün Fitokimyasal İçeriğinin Miktarsal Tayini için LC-MS/MS Metot Validasyonu

ÖZET: Bitkilerde 8 fitokimyasal bileşiğin (sinarin, kafeik asit, sirinjik asit, *p*-kumarik asit, *o*-kumarik asit, vanilik asit, ferulik asit, klorojenik asit) miktarsal tayini için kapsamlı bir LC-MS/MS metodu geliştirildi ve validasyon çalışmaları yapıldı. Geliştirilen analitik metot lineerite, gerçeklik (geri kazanım), gün içi ve günlerarası kesinlik (tekrarlanabilirlik ve tekrar üretilebilirlik), tespit ve tayin limitleri (LOD/LOQ) ve bağıl standart belirsizlik (% 95 güven aralığında (k=2)) gibi parametreleri içerek şekilde tam validasyon çalışmaları yapıldı. Kromatografik ayrım ters faz UHPLC sistemi ile yapıldı. Kütle dedeksiyonu ise her analit için negative veya pozitif iyonlaşma modları optimize edilerek üçlü kuadrupol kütle spektrometresi ile gerçekleştirildi. Analitlerin miktarsal tayini için çoklu reaksiyon görüntüleme (MRM) kullanıldı, moleküler iyonlar ve ilgili geçiş iyonları ise optimize edildi. Metot validasyonu sonrası, enginar (Cynara scolymus L.), brokoli (Brassica Oleracea var. Italica), karnabahar (Brassica Oleracea var. Botrytis) ve kenger gibi (Gundelia Tournefortii) bazı yenilebilir bitkilerin methanol ekstrelerinin fitokimyasal içerikleri geliştirilen ve valide edilen LC-MS/MS metodu ile tespit edildi. Analiz edilen bitkiler arasında enginar, açık ara farkla fenolik yönünden en zengini olarak belirlendi. Ek olarak, analiz edilen tüm bitkilerde klorojenik asit en bol bulunan bileşen olarak görüldü. Çalışılan örnekler analiz için seçilmiş bitkiler olsa da, geliştirilen LC-MS/MS metodu bitki dünyasındaki pekçok bitkiye uygulanabilirdir.

Anahtar kelimeler: Fitokimyasal, LC-MS/MS, metot validasyonu, yenilebilir bitkiler.

Cilt/*Volume:* 8, Sayı/*Issue:* 1, Sayfa/*pp:* 179-187, 2018
ISSN: 2146-0574, e-ISSN: 2536-4618 DOI: 10.21597/jist.407870

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Geliş tarihi / *Received:* 11.12.2017
Kabul tarihi / *Accepted:* 03.01.2018

INTRODUCTION

Natural polyphenols (i.e., phenolic acids, flavonoids, tannins) are compounds that are produced by plants and are involved in their defense mechanisms against biotic and abiotic stressors (Beckman, 2000). The consumption of polyphenol-rich plants such as vegatables and foods has been observed to be beneficial to human and animal health. To examplify, they are efficient in prevention of cardiovascular disease and cancer (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014; Feliciano et al., 2015; Turati et al., 2015). From the technological food point of view, these metabolites play an important role against oxidative damage, and thus prevent quality deterioration (Shahidi, 1997). However, the continuous request from consumers for a sustainable source and environmentally friendly production has increased scientific interest in the search for potential natural compounds in plant materials (Balasundram et al., 2006).

The globe artichoke (*Cynara scolymus* L.) being a herbaceous perennial crop is widely consumed in the Mediterranean area (Bianco, 2005). Its commercial production makes significant contribution to the agro-economy. Compared to other vegetables, globe artichoke embodies a high level of polyphenolic compounds (Brat et al., 2006; Lattanzio et al., 2009; Lombardo et al., 2010). Nonetheless, it is believed to be a promising source of biopharmaceuticals such as luteolin and mono-/di-caffeoylquinic acids, which are responsible for the therapeutic effects of the artichoke (Jun et al., 2007). Furthermore, antioxidant properties of the artichoke are thought to be related to its rich phenolic content (Mulinacci et al., 2004). For all these reasons, artichoke is regarded as a functional food. Several analytical methods have been reported on phenolic compound investigation in artichoke (Häusler et al., 2002; Sánchez-Rabaneda et al., 2003; Wang et al., 2003; Schütz, 2006; Lombardo et al., 2010).

Epidemiological studies have shown that consumption of *Brassica* vegatables such as broccoli and cauliflower reduces the risk of many noncommunicable diseases. This health-promoting effects of *Brassica* vegetables, are generally thought to originate from bioactive compounds such as glucosinolates (GSLs) and phenolic compounds (Latte et al., 2011; Sikora and Bodziarczyk, 2012; Kumar and Andy, 2012; Ares et al., 2013). Methods for identification and quantification of the main phenolic compounds in Brassicaceae vegetables have been generally based on HPLC coupled to DAD or MS detectors (Llorach, 2003; Vallejo, 2004; Bahorun, 2004; Ferreres et al., 2005; Harbaum et al., 2007).

Tumble thistle (*Gundelia Tournefortii* L.) being a member of compositae family is a medicinally important plant, important food source and native to the Asian-temperate zones of Western Asia, namely Turkey, Azerbaijan, Turkmenistan, Cyprus, Egypt, Iran and Israel (Coruh et al., 2007). There are some articles on the bioactivities and chemical contents of tumble thistle, though not many. Haghi et al.. (2011) investigated the caffeic acid derivatives in *Gundelia tournefortii* by HPLC.

The goal of this study was to develop and validate an LC-MS/MS method for the simultaneous and quantitative determination of phenolic compounds in vegatables. In this case, the phytochemical composition of methanolic extracts of some edible plants including artichoke (Cynara scolymus L.), broccoli (Brassica Oleracea var. Italica), cauliflower (Brassica Oleracea var. Botrytis) and tumble thistle (Gundelia Tournefortii).

MATERIALS AND METHODS

Chemicals

Cynarin (95%), caffeic acid (98%), syringic acid (95%), *p*-coumaric acid (98%), *o*-coumaric acid (97%), vanillic acid (97%), ferulic acid (99%), chlorogenic acid (95%), formic acid (98%), ammonium formate (99%) were purchased from Sigma Aldrich Co (Figure 1) . HPLC grade methanol was obtained from Merck and ultrapure water was obtained from Sartorious arium® pro ultrapure water system.

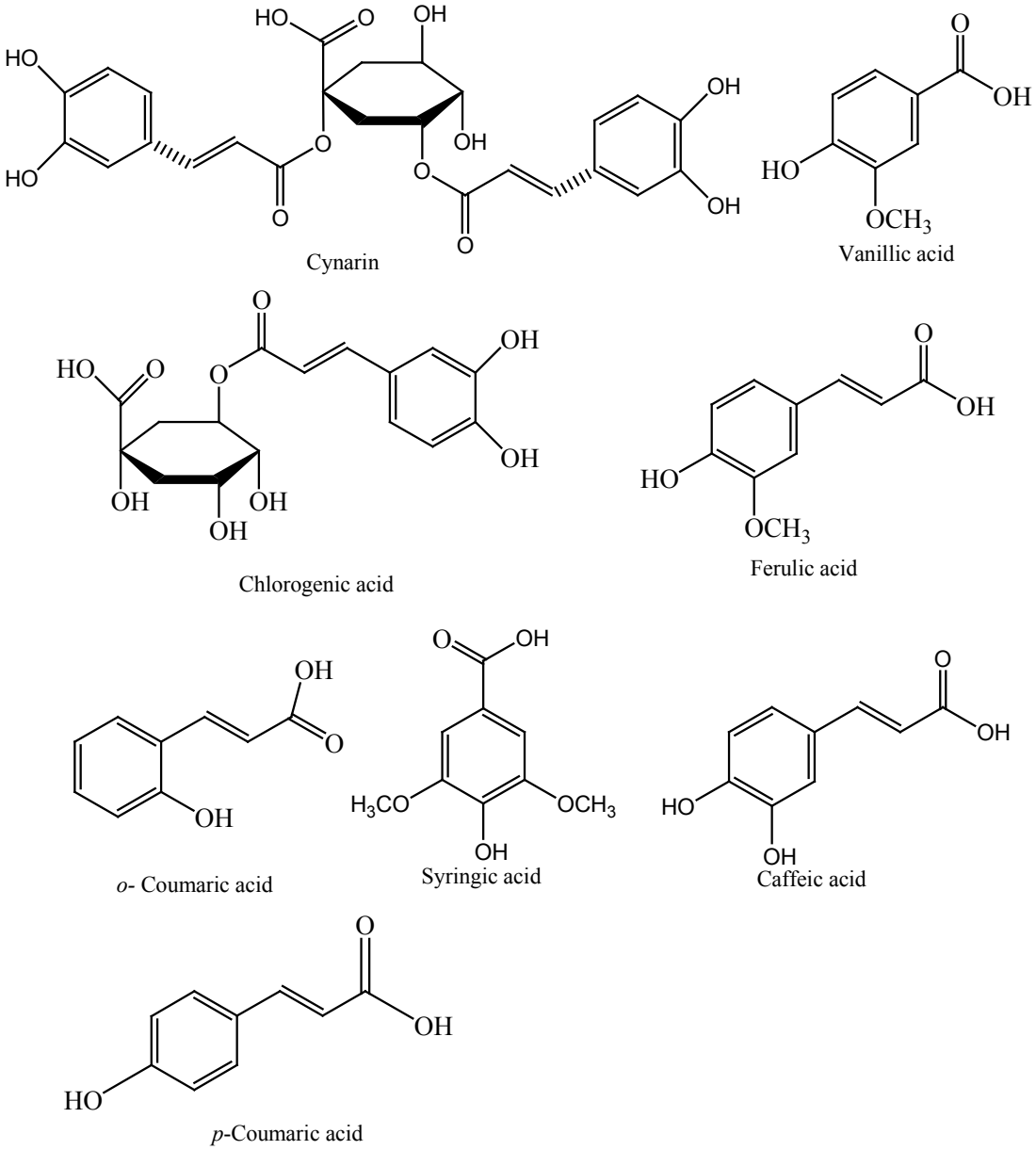

Figure 1. Phenolic compounds used in the LC-MS/MS method

1mg/mL stock solutions of 8 phenolic compounds were prepared from their solid forms. They were stored at -20°C when not studied. Afterwards, related calibration solutions were prepared by diluting these standard stocks.

Plant Material

Fresh artichoke, cauliflower, broccoli and tumble thistle samples, cultivated in Turkey, were purchased from a commercial local market. Then, the vegetables were seperated into different parts except from tumble thistle (artichoke; heart, outer stem, inner stem, outer leaves, inner leaves, cauliflower; stem, leaves, head, broccoli; outer stem, inner stem, leaves, head). After being purchased, vegatable samples were transported directly to the lab, washed with deionized water and stored at 4°C prior to be used.

Extraction Protocol

Firstly, fresh plant samples were divided into different parts, each part was homogenized by laboratory mixer and 10 grams of the samples were macerated with methanol three times (3×10 mL). After that, the extracts were filtered through 0,2 μ microfiber syringe filter and put into HPLC vial prior to injection.

Instrumentation and Chtomatographic Conditions

LC–MS/MS analyzes of 8 phenolic compounds were performed by using a Nexera model Shimadzu UHPLC coupled to a tandem MS instrument. The liquid chromatography was equipped with LC-30AD binary pumps, DGU-20A3R degasser, CTO-10ASvp column oven and SIL-30AC autosampler. The chromatographic separation was performed on a C18 reversed-phase ACE 3, 150× 4.6 mm analytical column. The column temperature was fixed at 30˚C. The elution gradient consisted of mobile phase A (water, 5 mM ammonium formate and 0.1% formic acid) and mobile phase B (methanol, 5 mM ammonium formate and 0.1% formic acid). The gradient program with the following proportions of solvent B was applied t (min), B%: (0, 10), (30, 90), (10,14) (90), (14.01, 20), (30). The solvent flow rate was maintained at 0.5 mL/min and injection volume was settled as 2 μL.

MS Instrumentation

MS detection was performed using Shimadzu LCMS 8040 model triple quadrupole mass spectrometer equipped with an ESI source operating in negative ionization mode. LC–MS/MS data were collected and processed by LabSolutions software (Shimadzu, Kyoto, Japan). The multiple reaction monitoring (MRM) mode was used to quantify the analyzes: the assay of investigated compounds was performed following two transitions per compound, the first one for quantitative purposes and the second one for confirmation. The optimum ESI conditions were determined as interface temperature; 350˚C, DL temperature; 250˚C, heat block temperature;400˚C, nebulizing gas flow (nitrogen); 3 L min^{-1} and drying gas flow(nitrogen); 15 L min^{-1}.

LC-MS/MS Method Validation And Quantitative Analysis

In this study, before the method validation process, chromatographic and mass spectrometric conditions were optimized. To do this; different columns, mobile phases (acetonitrile-water, methanol-water etc.), mobile phase additivies (ammonium acetate-acetic acid, ammonium formate-formic acid, ammonium hydroxide), column temeperatures and HPLC flow rates, were tried and the ones that serve for the best chromatographic seperation and mass ionization efficiencies were chosen for our method.

Following the optimization of chromatographic and MS conditions, the LC-MS/MS method was fully validated for the quantification of 8 phenolic compounds (cynarin, caffeic acid, syringic acid, *p*-coumaric acid, *o*-coumaric acid, vanillic acid, ferulic acid, chlorogenic acid) in edible plants. The performance characteristics of the developed method were determined by using standard solutions, spiked and non-spiked samples. Within this context, the developed method was fully validated in terms of linearity, accuracy (recovery), inter-day and intra-day precision (repeatability), limits of detection and quantification (LOD/LOQ) and relative standard uncertainty (U% at 95 % confidence level (k=2)). Parameters related to the LC-MS/MS method validation studies are given in Table 1.

Linearity

A calibration study was carried out over a wide concentration range in order to adapt to the broad spectrum of plants with significant phenolic content. The linearity studies were performed using external standard calibration curve with six concentration levels for each analyte, and each concentration level was assayed in triplicate. The developed method showed to be linear for all compounds, between the ranges of tested concentrations during the validation of the method with $R^2 \geq 0.9923$. The equations for the calibration curves and the determination coefficients (R^2) are shown in Table 1. Calibration graphs of the analytes are given in Figure 2.

Accuracy (Recovery) and Precision (Repeatability)

Accuracy and precision studies of the method were performed by standard addition to a selected vegetable (cauliflower stem) extract. For intra-day variability assessment, spiked samples were measured for six replicates within a single day, whereas spiked samples were examined in six replicates per day for three consecutive days to conduct inter-day assay. As a result of the intra-day and inter-day studies, recovery and %RSD values were calculated to determine the accuracy and precision (Table 1). The recovery was calculated with the following equation: recovery (%) = (amount found – original amount)/amount spiked × 100%.

Table 1. Analytical parameters that belong to the LC-MS/MS method ([a]RT: Retention time, [b]Mother ion(*m/z)*: Molecular ions of the standard compounds (m/z ratio), [c]R^2: Coefficient of determination, [d]RSD: Relative standard deviation, [e]LOD/LOQ (μgL^{-1}): Limit of detection/quantification, [f] U (%):percent relative uncertainty at 95% confidence level (k = 2))

No	Analytes	RT[a]	Mother ion (m/z)[b]	Fragment ions	Ion. mode	Equation	R^{2c}	RSD%[d]		Linearity Range (μg/L)	LOD/LOQ (μgL^{-1})[e]	Recovery (%)		U[f]
								Interday	Intraday			Interday	Intraday	
1	Cynarin	5.75	515.00	353.1-179.1	Neg	y=313.8x+4481.7	0.9989	0.0159	0.0166	50-1000	11.21/13.12	1.0098	1.0109	0.0321
2	Caffeic acid	6.87	179.10	135.1-134.1	Neg	y=5368.4x+29065.3	0.9994	0.0179	0.0178	10-200	2.58/3.61	1.0057	1.0040	0.0326
3	Syringic acid	6.96	197.10	182.1-123.1	Neg	y=17.8x-5334.0	0.9994	0.0042	0.0451	1000-20000	286.51/339.87	0.9998	1.0015	0.0099
4	*p*-Coumaric acid	8.10	163.10	119.1-93.0	Neg	y=458.9x+26917.5	0.9923	0.0094	0.0096	50-1000	8.62/9.92	1.0029	0.9960	0.0196
5	*o*-Coumaric acid	9.44	163.10	119.1-93.0	Neg	y=335.6x+1119.9	0.9999	0.0204	0.0132	50-1000	12.84/13.63	1.0058	1.0045	0.0308
6	Vanillic acid	7.01	167.1	152.1-108.1	Neg	y=11.9x-2594.3	0.9996	0.0050	0.0056	1000-20000	290.54/361.78	1.0011	1.0048	0.0122
7	Ferulic acid	8.14	193.1	178.1-134.1	Neg	y=18.1x-7913.3	0.9999	0.0064	0.0066	1000-20000	114.24/141.67	1.0016	1.0002	0.0143
8	Chlorogenic acid	5.60	353.00	191.1-85.0	Neg	y=1348.8x+4243.4	0.9996	0.0072	0.0052	10-200	2.35/2.81	0.9994	1.0006	0.0146

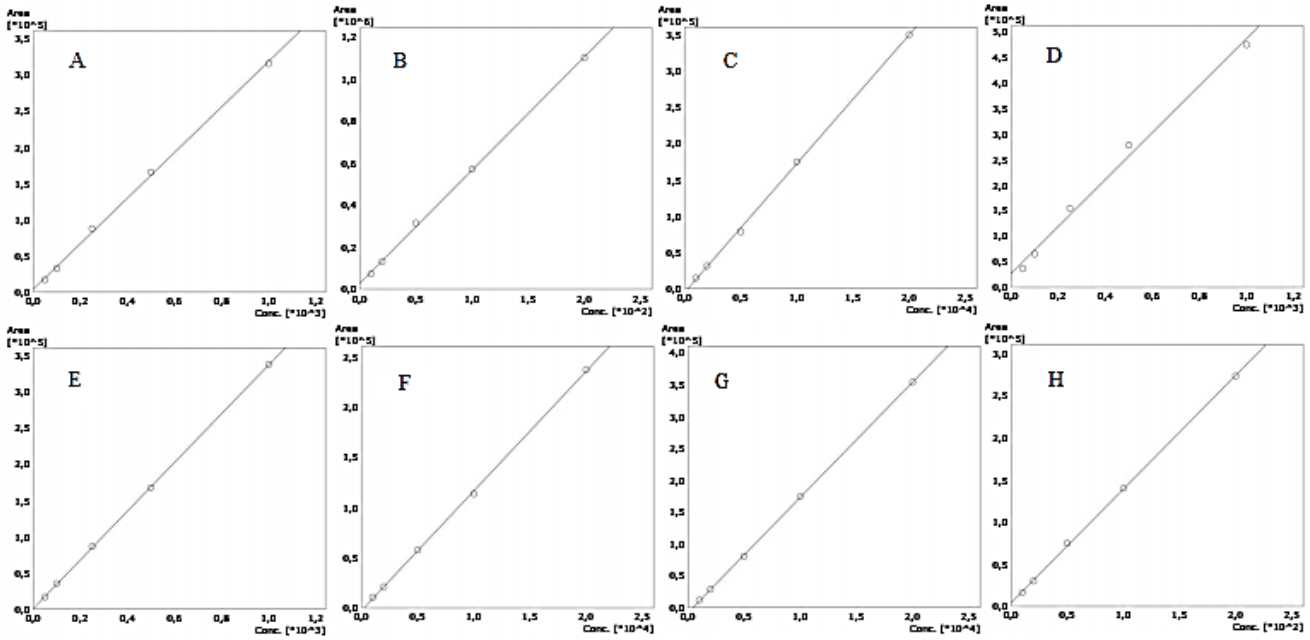

Figure 2. Calibration curves for the analytes studied in the LC-MS/MS method (A: Cynarin, B: Caffeic acid, C: Syringic acid, D: *p*-Coumaric acid, E: *o*-Coumaric acid, F: Vanillic acid, G: Ferulic acid, H: Chlorogenic acid)

Detection and Quantification Limits (LOD/LOQ)

In order to determine LOD and LOQ values for the analytes used in the LC-MS/MS method, analyte mixtrure were spiked at 10 identical samples prepared from a selected extract at the lowest concentration signaled by the standards and injected to the instrument. LOD and LOQ values detected for the analytes were as; 11.21/13.12 for cynarin, 2.58/3.61 for caffeic acid, 286.51/339.87 for syringic acid, 8.62/9.92 for *p*-coumaric acid, 12.84/13.63 for *o*-coumaric acid, 290.54/361.78 for vanillic acid, 114.24/141.67 for ferulic acid and 2.35/2.81 for chlorogenic acid. LOD and LOQ values were calculated according to the following equations (Table 1):

LOD=Mean+3×Standard Deviation

LOQ= Mean+10×Standard Deviation

Relative Standard Uncertainty (U^{95})

Standard uncertainties of the analytes were determined by the accuracy (recovery) and precision (repeatability) studies according to EURACHEM Guide (EURACHEM CITAC Guide, 2004).

RESULTS AND DISCUSSION

Quantitative analysis of Phenolic Compounds by LC-MS/MS

After method validation, the phytochemical composition of different parts of methanolic extracts of the studied fresh edible vegatables including artichoke (Cynara scolymus L.), broccoli (Brassica Oleracea var. Italica), cauliflower (Brassica Oleracea var. Botrytis) and tumble thistle (Gundelia Tournefortii) were investigated by the developed and validated LC-MS/MS method. The quantitation results and representative chromatograms (chromatograms of standard chemicals and)were given Table 2 and Figure 3, respectively.

According to the results, syringic, *o*-coumaric and ferulic acids weren't detected in any parts of the extracts. In addition, chlorogenic acid was the most abundant phenolic compound present in all extracts. Nevertheless, heart (19651.38 μg g^{-1} fresh plant) and inner leaves (14738.88 μg g^{-1} plant) of artichoke sample contained the highest

amounts of chlorogenic acid. Similarly, tumble thistle (6863,64 μg g^{-1} fresh plant), outer and inner stems of artichoke (5162,88 and 3747,32 64 μg g^{-1} fresh plant, respectively), outer leaves of artichoke (2806,88 μg g^{-1} fresh plant) and head parts of broccoli (2075,66 μg g^{-1} fresh plant) were rich in terms of chlorogenic acid. On the other hand, chlorogenic acid content of cauliflower-stem and broccoli-inner stem etxracts were quite low.

Moreover, the heart (168.42 μg g^{-1} fresh plant) and inner leaf (635.63 μg g^{-1} fresh plant) parts of the artichoke are the only extracts containing vanillic acid. Inner stem part of artichoke (58.83 μg g^{-1} fresh plant) was the most abundant extract containing caffeic acid. Inner leaves of artichoke (21.95 μg g^{-1} fresh plant), tumble thistle (25.52 μg g^{-1} fresh plant) and head of broccoli (25.86 μg g^{-1} fresh plant) were also rich in caffeic acid compared to other extracts. Besides, head of cauliflower (135.20 μg g^{-1} fresh plant) was the richest extract containing *p*-coumaric acid. When it comes to cynarin content, artichoke parts were the only extracts containing cynarin. Inner leaves (157.81 μg g^{-1} fresh plant), outer leaves (106.22 μg g^{-1} fresh plant) and heart (82.45 μg g^{-1} fresh plant) parts of the artichoke were the most abundant extracts containing cynarin.

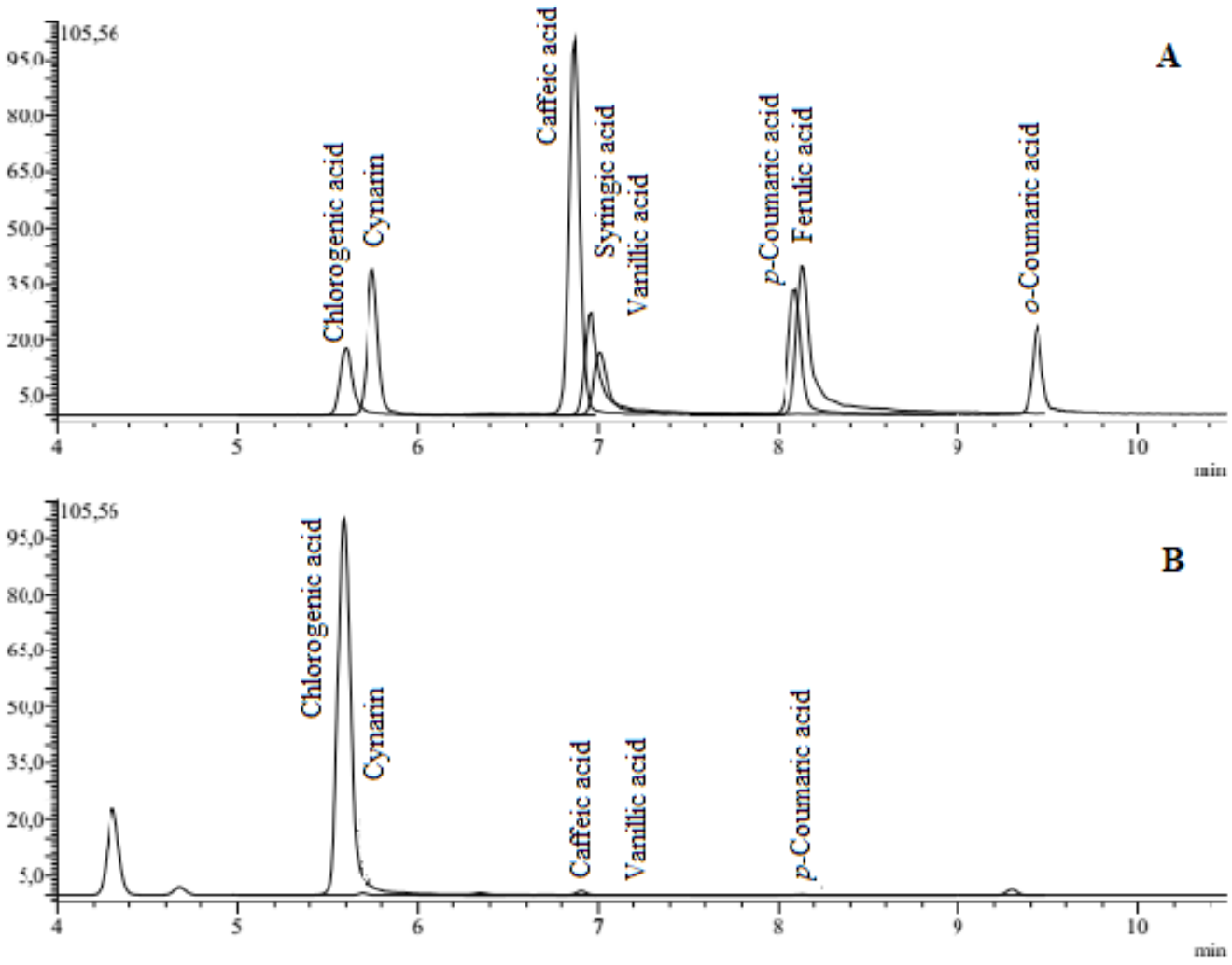

Figure 3. LC-MS/MS TIC chromatograms of A: Standard analytes, B: Artichoke inner leaf sample

Table 2. Quantitative results (μg analyte/g plant) for the phenolic analysis of samples by LC-MS/MS

Samples	Cynarin	Caffeic acid	syringic acid	*p*-Coumaric acid	*o*-Coumaric acid	Vanillic acid	Ferulic acid	Chlorogenic acid
Artichoke-heart	82.45	71	N.D.[a]	4.07	N.D.	168.42	N.D.	19651.38
Artichoke-outer stem	24.41	13.66	N.D.	6.5	N.D.	N.D.	N.D.	5162.88
Artichoke-inner stem	31.36	53.83	N.D.	5.48	N.D.	N.D.	N.D.	3747.32
Artichoke-inner leaf	157.81	21.95	N.D.	49.67	N.D.	635.63	N.D.	14738.88
Artichoke-outer leaf	106.22	3.96	N.D.	38.6	N.D.	N.D.	N.D.	2806.88
Tumble thistle	N.D.	25.52	N.D.	9.17	N.D.	N.D.	N.D.	6863.64
Cauliflower-stem	N.D.	0.84	N.D.	32.29	N.D.	N.D.	N.D.	45.37
Cauliflower-leaf	N.D.	1.52	N.D.	15.97	N.D.	N.D.	N.D.	326.61
Cauliflower-head	N.D.	1.13	N.D.	135.2	N.D.	N.D.	N.D.	524.99
Broccoli-head	N.D.	25.86	N.D.	14.91	N.D.	N.D.	N.D.	2075.66
Broccoli-leaf	N.D.	3.32	N.D.	9.4	N.D.	N.D.	N.D.	1675.51
Broccoli-outer stem	N.D.	1.82	N.D.	9.64	N.D.	N.D.	N.D.	245.58
Broccoli-inner stem	N.D.	0.88	N.D.	15.89	N.D.	N.D.	N.D.	20.68

[a] N.D.: Not detected

In a previous study on Brazilian vegatables, it was reported the caffeic acid content of artichoke and broccoli (Tiveron, 2012). Furthermore, Haghi et al. reported the chlorogenic acid and caffeic acid contents of tumble thistle in their study (Haghi, 2011). In another study, Negro et al. reported the chlorogenic acid and caffeic acid contents in artichoke plant tissues (Negro, 2012).

CONCLUSION

A rapid LC-MS/MS method was developed for the simultaneous and quantitative determination of eight phenolic compounds in plant tissues. The developed LC-MS/MS method was fully validated in terms of linearity, accuracy (recovery), inter and intra-day precision (repeatability), limits of detection and quantification (LOD/LOQ) and relative standard uncertainty (U% at 95% confidence level (k=2)). In the current syudy, the phytochemical composition of methanolic extracts of different parts of some edible plants including artichoke (Cynara scolymus L.), broccoli (*Brassica Oleracea var. Italica*), cauliflower (*Brassica Oleracea var. Botrytis*) and tumble thistle (*Gundelia Tournefortii*) were investigated. According to the results, syringic, *o*-coumaric and ferulic acids weren't detected in any parts of the extracts. Additionally, chlorogenic acid was the most abundant phenolic compound present in all extracts. Nevertheless, heart (19651.38 μg g^{-1} fresh plant) and inner leaves (14738.88 μg g^{-1} plant) of artichoke sample contained the highest amounts of chlorogenic acid.

ACKNOWLEDGEMENT

Authers would like to thank Dicle University Science and Technology Research and Application Center (DUBTAM) for opening its laboratory facilities during the study

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