Determination of Antioxidant Activity of Selected Vegetables Grown in Iran under Reversed Phase Conditions with UPLC

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ABSTRACT
This study describes the determination of some phenolic compounds in four different vegetables of Kerman in Iran including Carrots, Celery, Lettuce and red cabbage. The phenolic compounds analyzed were (Ascorbate, Ferulic acid, Naringin) using reverse phase high performance liquid chromatography (RPUPLC). The results of analysis showed in vegetables ranged between 90 and 1080mg / kg. A high and significant correlation between antioxidant activity and total phenolic content was determined in vegetables (r² = 0.9461 P < 0.06). However, flavonoid content was not significantly correlated with antioxidant activity in vegetables. It was observed that total phenolic content is the major contributor to the antioxidant activity of vegetables.

Key words: Antioxidant activity; total phenolics; total flavonoids; vegetables; Reverse phase; UPLC

INTRODUCTION
Interest in the role of antioxidants in human health has prompted research in the fields of food science and horticulture to assess fruit and vegetable antioxidants (Kalt et al., 1999). The majority of the antioxidant capacity of a fruit or vegetable may be from compounds such as flavonoids, isoflavones, flavones, anthocyanins, catechins and isocatechins rather than from vitamins C, E or β-carotene (Wang et al., 1996; Kähkönen et al., 1999). Many of these phytochemicals may help to protect cells against the oxidative damage caused by free radicals (Wada and Ou, 2002). Fruit and vegetable antioxidants play an important role in reducing the risk of degenerative diseases such as cardiovascular disease, various cancers and neurological diseases (Kalt et al., 1999). There are approximately 5000 known plant phenolics and model studies have demonstrated that many of them have antioxidant activity (Robards et al., 1999). The antioxidant activity of phenolics is mainly because of their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Rice-Evans et al., 1995). Their antioxidant activity is generally based on the number and location of hydroxyl groups present as well as the presence of a 2-3 double bond and 4-oxofunction (Rice Evans and Miller, 1998). The flavonoids, a large family of low molecular weight polyphenolic compounds, include the flavones, flavonols, flavonones, isoflavones, flavan-3-ols and anthocyanins (Stewart et al., 2000). Although flavonoids are generally considered non-nutritive agents, interest in these substances has risen because of their possible effects on human health (Hertog et al., 1992). In addition to their antioxidant activities, flavonoids inhibit enzymes such as prostaglandin synthase, lipoxygenase and cyclooxygenase, closely related to tumorigenesis, and may induce detoxifying enzymes such as glutathione Stransferase (Lee et al., 1995). Many kinds of flavonoid have been reported in fruits and vegetables and their types and contents vary with cultivar and maturation (Hertog et al., 1992). All of these aspects explain the increasing interest in fruit phenolics that has been manifested in the past few years. In this context, a large number of plant sources including many fruits and vegetables have been explored for their antioxidant potential. Therefore, the main objective of this study was to determine the antioxidant activity of different fruits and vegetables grown in Turkey. Another aim was to evaluate whether total phenolic and flavonoid...
contents of samples are correlated with antioxidant activity. A diet rich in vegetables (more than 5 servings per day) is recommended along with fruits and whole grains; an epidemiological study found that a diet of this composition has a negative association with the risk of chronic diseases. Antioxidant vitamins in vegetables are some of the important nutrients besides other vitamins, minerals, flavonoids and phytochemicals, which have been reported to contribute to health. Our local markets offer a variety of vegetables ranging from leafy to tubers for consumption. (Tee et al., 1997).

Beside the conventionally grown vegetables, currently, organically grown foods are gaining popularity among consumers, health educators, farmers and food retailers. Many consumers believe that organically grown vegetables are of better quality, healthier and more nutritious than conventionally grown ones.

This is due to a number of reasons which may vary from country to country such as safety, effect of environment, flavour, freshness, health benefits and nutritional value (Bourn & Prescott, 2002). In this field, high performance liquid chromatography (HPLC) is one of more promising and more used techniques, either by direct determination or by the analysis of derivatized products. Most of procedures developed until now for food and beverage analysis utilize either reverse phase partition chromatography (Czajkowska and Jaroniec, 1997; Fransson and Ragnarsson, 1998) or ion exchange chromatography (Casella and Gatta, 2001; Guillen et al., 1998; Linget et al., 1998) with a refractive index (RI), UV spectrophotometric, conductimetric or electrochemical detection (Buchberger, 2000).

The separation of antioxidants with liquid chromatography and their quantitative determinations are extremely difficult because there is no difference between their structural similarities and spectral characteristics. Besides, pKa values of most of the antioxidants are rather similar and this situation limits the usage of pH for chromatographic separation (Aktas et al., 2005).

**MATERIALS AND METHODS**

**Vegetables**

Tetra types of green vegetables were selected based on popular consumption among Iran. Conventionally grown vegetables (400 g) were purchased from a local wet market in Kerman. Convenience sampling was used to obtain the samples. Fresh vegetables (carrots, celery, lettuce and red cabbage) were purchased fresh from local markets in Kerman in summer. All vegetables were washed and grated before extraction. Healthy vegetables were selected randomly for uniformity of shape and color.

**Preparation of samples**

Upon arrival at the Department of Nutrition and Health Sciences laboratory, the fresh and healthy vegetables were immediately washed under tap water and excessive water dripped off. Edible portions (100 g) of the vegetables were cut into small pieces and homogenised using a blender (National; model MX-291N) for 2 min. The homogenised sample was transferred into an air-tight container and kept at -20°C before vitamin analysis. Determination of Vitamin C and Phenolic acids in Green Vegetables. The vegetables juice was extracted and careful hand-squeezing to obtain the juice. The juice was passed through a strainer to remove pulp. The freshly squeezed juice was centrifuged at 3000 rpm for 10 min and twice, the supernatant was diluted 1:5. The dilutions were membrane filtered (0.20 μm) before injection.

**Analysis by UPLC**

Antioxidants in the samples test solution were separated by reversed phase chromatography on a 150 mm×4.6 mm i.d., 5 μm particle ZORBAX Eclipse XDB-C18 analytical column, of which was detected by absorbance and quantified with external calibration graph. For the simultaneous detection of the tetra analytes, the detector was set at λ=254 nm for ascorbic acid and λ=214 nm for the other organic acids. This setting was chosen since ascorbic acid has its maximum optical absorbance close to 254 nm.

The UPLC analysis was performed with an Agilent 1200 series system. Integration, data storage and processing were performed by Chemstation software. The determinations were made in isocratic conditions, at ambient temperature, using a mobile phase made of 0.2 % acetonitrile and 50 mM phosphate solution (dissolve 6.8 g potassium dihydrogen phosphate in 900 ml water; the pH value should be adjusted to pH =2.8 with phosphoric acid and then filled to 1000 ml with water) filtered through a polyamide membrane (0.2 μm) and degassed in a vacuum. The flow rate of the mobile phase was 1.2 ml/min for all the chromatographic separations. The separation column was balanced with mobile phase until the baseline was stabilized. Sample injections were made at this point. The volume injected was 5 μl for either prepared sample or standard solution.

**Determination of phenolic compounds**

The phenolic compounds (gallic, catechin, caffeic, chlorogenic, ocomaric, p-coumaric, ferulic, ciringic, vanillic, quercetin, and rutin acids) were determined using the HPLC separation method described by Rodriguez Delgado et al. (2001). In three replicates, about 100g of samples was fragmented and 5mL fruit juice from each sample was transferred to centrifuge tubes. The samples were mixed homogenously then diluted 1:1 with distilled water and centrifuged at 15000g for 15 minutes. The supernatant were passed through 0.45 mm membrane filter (Millipore Millex-HV Hydrophilic PVDF,
Millipore, USA), then injected into HPLC system. The Chromatographic separation in Agilent 1100 series HPLC was took placed in DAD detector (Agilent, USA) with 250 x 4.6 mm, 4µm ODS column (HiChrom, USA). The following solvents in water with a flow rate of 1 mL min\(^{-1}\) and 20 µL injection volume were used for spectral measurements at 254 and 280 nm: as mobile phase solvent A, methanol-acetic acid-water (10:2:88) and Solvent B, methanol-acetic acid-water (90:2:8) (Table 1).

**Determination of total antioxidant activity**

For the standard trolox equivalent antioxidant capacity (TEAC assay, ABTS [29,2-azinobis-(3-ethylbenzothiazoline-6sulfonic acid)] was dissolved in acetate buffer and prepared with potassium peroxide, as described by RiceEvans et al. (1995) and Ozgen et al. (2006). The mixture was diluted in acidic medium of 20 mM sodium acetate buffer (pH 4.5) to an absorbance of 0.700 ± 0.01 at 734 nm for longer stability (Ozgen et al., 2006). For the spectrophotometric assay, 3 mL of the ABTS+ solution and 20 µL of fruit extract were mixed and incubated for 10 min and the absorbance was determined at 734 nm.

**RESULTS AND DISCUSSION**

In Fig. 1 the chromatogram of ascorbic acid and naringin in the standard solution and real samples were given. The linearity of the method was evaluated according to area response. Selected wavelength for ascorbic acid, retention time, and concentration ranges of linear response, correlation coefficients and detection of limit were summarised in Tab. 1.

The detection limit (LOD) could be defined as the smallest peak detected with a signal height three times that of the baseline, while the limit of quantification (LOQ) referred to the lowest level of analyte which could be determined with an acceptable degree of confidence.

In the present work, detection limits were estimated according to the hypothesis that a peak, to be detected, should have a signal-to-noise ratio >3.

Precision was tested on ten replicated analyses of independent preparations of vegetables. The RSD value

| Tab. 1. Wavelength, retention time, concentration range of lineal response, correlation coefficient and detection limit for standards. | Tab. 2. Ascorbic acid and phenolic acids content (gr/l) of vegetables. |
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| Analyte | \(\lambda_{\text{nm}}\) | RT(min) | Concentration range (mg/l) | Correlation coefficient(\(r^2\)) | Detection limit (mg/l) | Vegetables | Ascorbate | Ferulic acid | Naringin |
| Ascorbate | 254 | 3.2 | 0.5-200 | 0.9990 | 0.20 | Carrots | 1.08 | 0.57 | 1.60 |
| Ferulic acid | 283 | 4.1 | 0.5-100 | 0.9994 | 0.04 | Celery | 0.98 | 0.82 | 0.21 |
| Naringin | 283 | 1.12 | 5.0-200 | 0.9995 | 0.07 | Lettuce | 0.68 | 0.92 | 0.37 |
| red cabbage | 0.18 | 0.47 | 0.09 |

**Fig.1:** Chromatogram of ascorbic acid in the standard solution (a), naringin standard (b), Red cabbage (c) and Celery
was % indicating that the method was precise with a high degree of repeatability. The recovery of ascorbic acid from vegetables ranged from 97.2 to 103.6%. The amount ascorbate, ferulic acid and naringin found in vegetables were shown in Tab. 2.

CONCLUSION

The present study was the first comprehensive investigations to determine the phenolic contents and antioxidants in vegetables grown in Kerman ecological conditions. Therefore, this initial study would be more convenient to organize further researches in the coming periods. Based on the results obtained from the present study, employment of the cultivars (having more phenolic substances) in vegetables processing technology might contribute to the effective outcomes. It was seen that the Carrots was in the forefront because which Naringin has analgesic, anti-allergenic, anti-asthmatic, antibacterial, immunostimulant, antiviral, antiseptic and cancer-preventive effects in terms of health. Moreover, had higher red cabbage values in terms of antioxidant activity.

This work is a contribution to the development of a rapid and precise UPLC procedure for quantitative determination of total phenolic in vegetables. ascorbate ,Ferulic acid and Naringin have been eluted from the column within 4 minutes. The method could successfully used to quantify antioxidants in natural vegeatbles.

It was observed that the antioxidants present in vegetables were species, cultivar and horticultural practice dependent and could be considered as an active parameter for authenticity determination.

REFERENCES


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