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Fast High Performance Liquid Chromatography and Ultraviolet Method for Determination of Phenolic Antioxidants in Fresh Rosemary Leaves.

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Abstract
An improved reversed phase HPLC method is reported for the determination of rosemary's principal phenolic antioxidants, caeffic acid, naringin ,rosmarinic acid ,cirsimaritin, carnosol and carnosic acids, providing a fast and simultaneous quantitative determination for these compounds , The analysis can be accomplished within 10 min under isocratic conditions with 0.1% phosphoric acid-60% acetonitrile as the mobile phase at a flow-rate of 1.5 ml/min, with UV detection at 280 nm. Using efficient reversed phase , Hypersil 5H,C-18 (50x 4.6 mm I.D) column, 3µm particle size, The analysis was performed with fresh methanolic extractions of *Rosmarinus officinalis* leaves . To quantify the amount of antioxidants in a fast and reproducible way by means of UV–vis absorption measurements, excellent linearity were obtained for all studies standards ,with coerelation coeficients of R value 0.964 and standared deviation were (SD=0.551), the detection limits were less than 0.1 µg/l. the result observed that the main constituents in the rosemary leaves were carnosic acid and rosemarinic acid with low concentration of caffeic acid, naringin, cirsimaritinand carnosol. This UV–vis methodology can be extended to the determination of other compounds and herbs constituents of phenolic antioxidants .
Keywords:*Rosmarinus officinalis*; antioxidants ; HPLC analysis

Introduction

An antioxidant may be roughly defined as “any substance that when present at low concentrations,will be significantly delays or inhibits oxidation of oxidizing compounds". There are two basic categories of antioxidants, natural and synthetic, the second ones have been found to cause long-term toxicological effects, including carcinogenicity [1,2] Consequently, natural herbs antioxidant have been used as flavourings, beverages, repellents, fragrances, cosmetics and for various medicinal applications.

Nowadays, the Rosemary (*Rosmarinu sofficinalis* L.), for example, is an economically important herb known not only as a source of essential oils but also for its natural antioxidants [1-,3]. The presence of diterpenes such as carnosic acid and carnosol, these two natural compounds with high antioxidant activity, has been reported [3,4] and several flavonoids and phenolic compounds such as hispidulin, cirsimaritin, apigenin, genkwanin, naringin, caffeic acid and rosmarinic acid are also present in rosemary extracts [4,5]

The antioxidant activity of rosemary extracts depends on their composition. There are many reports that analysed and determined their antioxidant capacity by various methods using lipid and aqueous systems. In lipid systems, extracts with higher diterpene content were the most effective [6,7]. while in aqueous systems rosmarinic acid exhibited the highest antioxidant activity [6]. Rosmarinic acid, as one of the most abundant and powerful natural antioxidant in various important *Lamiaceae* species,

The extraction of natural antioxidant to replace synthetic food preservatives

has become increasingly more important [8,9] There is also a growing number of potential uses and new commercial products being obtained from materials traditionally used as condiments. Currently, most of the interest is focusing on phenolic antioxidants of herbal origin. , among these are carvacrol and thymol from oregano (*Origanum vulgare*) thymol from thyme (*Thymus vulgaris*)[10] carnosic acid (CA) from rosemary [11].

Several reports have been published analysing the distribution of rosmarinic and/or carnosic acids during growth and vegetative development of rosemary leaves growing conditions were studied simultaneously on both rosmarinic and carnosic aids [12-15].

The plant source antioxidants had been analyzed by using different detection and quantitative measurement methods. The previous method include the kinetics of the reaction of (2,2'-diphenyl- 1-picrylhydrazyl DPPH) and and 2,2'-azinobis (3-ethylbenzo- thiazoline-6-sulfonic acid) radical cation (ABTS+•) with flavonoids were studied in detail by Butkovic at al[12] During recent years, (DPPH•)) the online HPLC–DPPH•–ABTS• methods were succesfully used for the analysis of sweet grass [13] thyme [14] various *Salvia* species [15]borage [16] apples [17] coffee [18] *Geranium macrorrhizum* [19]), *Potentila fruticosa* [20] *Mentha* species [21] and selected *Lamiaceae* species [22] But these method is time consuming ,when we have alot of sample for analysis.

In this work, we present a fast chromatographic methodology using short chromatographic column with fine particle size 3 µm, whic facilitate a clean and inexpensive spectrophotometric analytical model intended to analyze simultaneously different phenolic antioxidants present in *R. officinalis*.

Material and Methods

Chemicals

All solvents used in the experiments were HPLC grade and were purchased from Fisher Scientif ic (UK). The standards caffeic acid, rosmarinic acid carnosic acid ,carnosol , naringin and cirsimaritin were purchased from Sigma-Aldrich Company Ltd. (UK).

Rosemary leaves were collected from the farm of college of agricutre ,Baghdad.

Extraction method

Fresh plant material (1 g) was ground in liquid nitrogen and extracted three times with 20 ml of HPLC methanol for 15, 10 and 5 min at room temperature (RT), in a ultrasonic bath. The combined extracts were evaporated to dryness under stream of liquid nitrogen at room temperature .

The residues were dissolved in 1 ml of methanol. The mixtures were filtered through paper filter MN 615 (Macherey-Nagel, Düren, Germany), and the resulting liquid extracts were stored in a freezer at –20°C under nitrogen until analysis. The analysis was performed within 1 month after the storage .

HPLC analysis

Before the HPLC analysis all the sampleswere filterd and aliquots of 20 µl were injected into a reverse phase Hypersil H5 ODS column (50 × 4.6 mm i.d.). 3µm particle size ,A Shimadzu LC-10AT vp binary HPLC system equipped with System controller coupled with a SPD-10A VP spectrophotometer detector were used. Separation and quantification were achieved at 25ºC by using isocratic conditions with 0.1% phosphoric acid-60% acetonitrile as the mobile phase at a flow-rate of 1.5 ml/min .

Identification of individual compounds was based on the comparison of the actual retention time to those of reference authentic standards.

The total µg ml-1 of antioxidants used was calculated from the total phenolic content of the extracts (the sum of caffeic acid, rosmarinic acid , naringin, cirsimaritin, carnosol, and carnosic acid concentrations), quantified by the HPLC.

Results and Discussion

HPLC analysis of the methanol extract of the *rosemery leaves* resulted in the separation of carnosic acid and rosmarinic acid as the major compounds, together with several minor components caffeic acid ,naringin, cirsimaritin and carnosol , in less than 10 minute as shown in typical chromatogram of standard (Fig 1 A) and extracted sample as shown in (Figure 1 B) was identified by comparison the retention time of authenic standared witch that of the sample, all compound measured at 280 nm. Table 1 below show the elution order of eluted compounds with the concentration obtained as amean ±standerd deviation.

The results observed that , the highest concentration belong to csrnosic acid and rosemarinic acid which is the major antioxidanta in rosemery leaves while the other antioxidant represnts 7.73 % of antioxidant .

Table 1:

Identified compounds and concentration levels in rosemary leaves. Retention times are expressed in minutes,and concentrations in mg g-1 fresh weight . The data represent the mean ± standard deviation for n = 3 different determinations.

sequence	compounds	Etention time min	Mean mg/gm concentration±SD
1	Caffeic acid	3.15	0.12
2	Naringin	5.12	0.08
3	Rosmarinic acid	5.93	2.34
4	Circimaritin	7.10	0.09
5	Carnosol	8.09	0.98
6	Carnosic acid	9.06	12.81
	Total		16.42 mg/gm

HPLC analysis of rosmarinic and carnosic acids variations in rosemary plants and numerous phenolics, flavonoids, and diterpenes have been reported in rosemary extracts [23-26], in our study only, caffeic acid, rosmarinic acid, naringin, cirsimaritin, carnosol, and carnosic acid were present in sufficient amount to be identified and quantified by this methods. For quantification purposes and to guarantee full extraction and reproducibility of the method, one sample was subjected to a set of extraction conditions using different amounts of material, solvent, and extraction times The best results were obtained by using a three-fold extractionas it was described in extraction procedure. In addition, the quantification of rosemary compounds at 280 nm, a wavelength at which all compounds were detected, to made routine analysis more feasible allowing the quantification of all compounds in only one HPLC run, even when the photodiode array detector was not available.

Concentrations of the six compounds from rosemary extracts were studied by using the optimum extraction and HPLC methodology. Rosmarinic and carnosic acids were the most abundant compounds followed by caffeic acid ,carnosol, circimaritin, and naringin as predicted in (Table 1). Additionally, when the plant

distribution of these two main components was studied in rosemary extracts, rosmarinic and carnosic acid was found in leaves with high abudant which can made usfel for medical applications.

A **B**

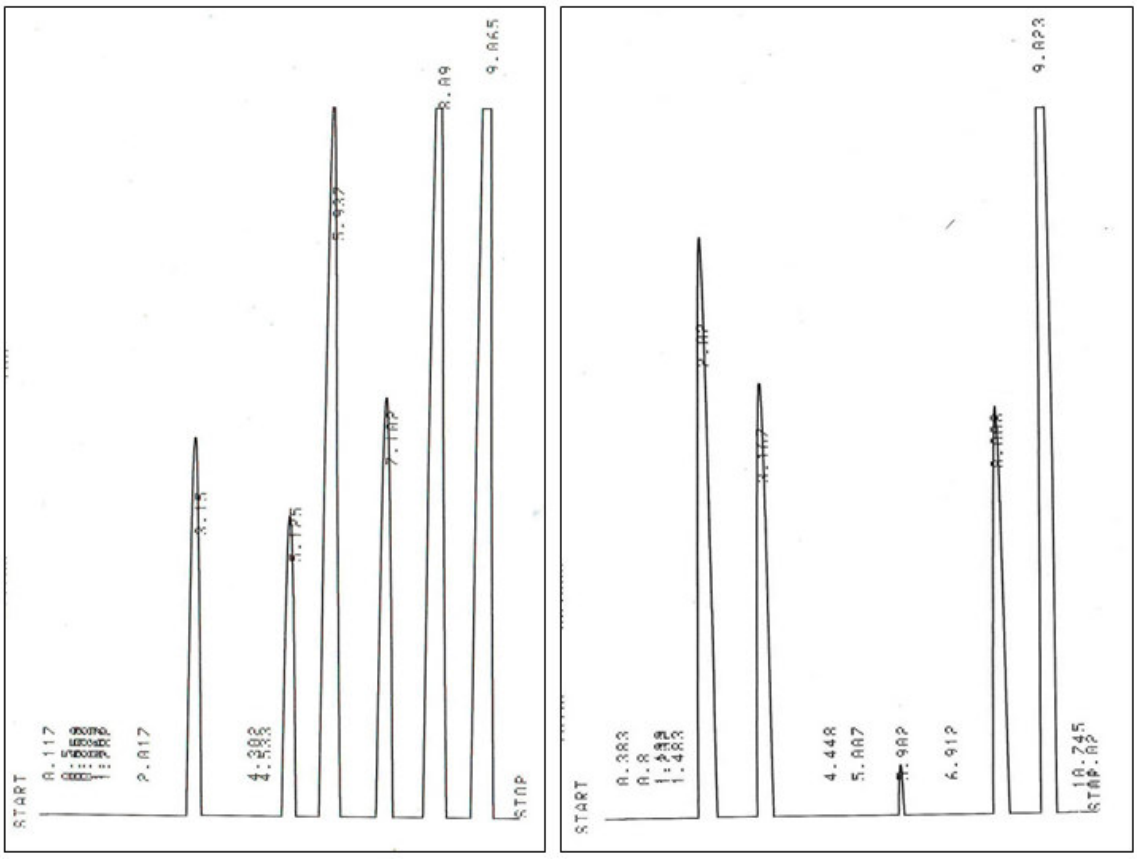

Fig 1: Reversed Phase HPLC Separation of antioxidant Mixture under optiminim condition:
Mobile Phase= 0.1% Phosphoric acid 608Acetonitrile
Column, Hypersil Hs ODS/(50X 46 µm.ID) 3µm particle size.
Temperature: 30 Cº
Flow rate: 1.5 µl/ min
Detection: UV set at 280 nm
Injection volume: 20 µL
A: Standard mixture , B: Rosemary leaves extract

Conclusions

The reported chromatographic method employs a solid phase column and permits a very fast separation of carnosic acid, carnosol and rosmarinic acid, in less than ten minutes of analysis.

This fast procedure shows a very good resolution and was developed to perform simultaneous determination of lipophillic and hydrophillic antioxidants present in the sample. An UV–vis method for quanitative evalution the content of the compounds of interest with known UV-Vis is possible in a fast and reproducible form.

Samples of the same nature (extractions of dried leaves, for example) must be employed in the calibration and in the subsequent measurements, in order to have a similar profile for the unknown compounds. The procedure is appropriate for obtaining an accurate routine and near field analysis of compound content during harvest, or raw material quality control of extract production, yielding results almost instantaneously once the calibration curve is ready.

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