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Reversed phase high performance liquid chromatography method for quantification of antioxidants in fresh rosemary leaves

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ABSTRACT

Fast and accurate HPLC method is adopted for determination of eight rosemary's phenolic antioxidants, (vanillic acid, caffeic acid, rosmarinic acid, naringin, hispidulin, cirsimaritin, carnosol and carnosic acid) from fresh leaves by multi-solvents extractions of *Rosmarinus officinalis* ,by dissolving 1 gm in water: methanol: 2-propanol (each one contained 0.1% *O*-phosphoric acid) with ratio of (60:30:10 , v/v), these ompounds were identified and quantified, the main predominant antioxidants in rosmarey leaves were rosmarinic acid(3.927 ± 0.129 mg/gm) and carnosic acids (13.79 ± 0.598 mg/gm) followed by Naringina (0.848 ± 0.034 mg/gm) and, carnosol (0.775 ± 0.0354 mg/gm) using a mini ,(solid phase extraction SPE) column for pre-concentration and purification the extract. The analysis was performed on reversed phase (50x 4.6 mm I.D), 3µm particle size C-18 DB column, mobile phase was,(water: methanol: 2-propanol each one contained 0.1% *O*-phosphoric acid), using gradient program of multi-solvent as mobile phase , detection UV set at 285 nm, flow rate 1.2 ml/min. the separation occurred under the optimum separation condition for both standard and sample to quantify the amount of antioxidants in a fast and reproducible way by means of UV–VIS absorption measurements.

Keywords: Rosmarinus officinalis; c antioxidants, HPLC, UV detection

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INTRODUCTION

An antioxidant may be defined as "any substance that when present at low concentrations, lower than the oxidizable compound to be protected, significantly delays or inhibits its oxidation". Two basic classification of antioxidants, natural and synthetic, the second ones have been found to cause long-term toxicological effects, including carcinogenicity [1].therefore , there is an increasing interest in finding naturally occurring antioxidants form the herbs have been used as flavourings, beverages, repellents, fragrances, **c**osmetics and for wide applications in medicinal fileds., the interest in herbs has considerably increased, particularly as a natural source of antioxidants for the food and pharmaceutical industries. Rosemary (*Rosmarinu officinalis* L.), for example, is an economically important herb known not only as a source of essential oils but also for its natural antioxidants,[1-4]. The presence of diterpenes such as carnosic acid and carnosol, two natural compounds with antioxidant activity, has been reported previously [5-7] and several flavonoids and phenolic compounds such as hispidulin, cirsimaritin, apigenin, genkwanin, naringin, caffeic acid and rosmarinic acid are also present in rosemary extracts [3,4].

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 [8] while in aqueous systems rosmarinic acid exhibited the highest antioxidant activity [7-9].

Several reports have been published analysing the distribution of rosmarinic and/or carnosic acids during growth and vegetative development of rosemary leaves [4,9-11].

Rosmarinus belong to Labiatae family, which have been worldwide used in traditional medicine for weakness, depression, memory enhancement, blood circulation improvement, strengthening of fragile blood vessels, [12] inflammation, infection, [13] indigestion and gastritis.[14]. Researchers have proved that these plants are source of compounds with high antioxidant properties, [3] anti-inflammatory,15] anti-allergic,[16] anti-depression,[17] anti-hyperglycemic, [18] and antimicrobial,[19-21] properties. Most Rosmarinus extract activities are mostly related to their phenolic compounds content especially rosmarinic acid , an ester of caffeic acid which was isolated for the first time from *Rosmarinus officinalis* L. leaves and later found in other species of Labiatae family in different quantities, the most important constituent of Rosmarinus is rosmarinic acid due to their interesting properties which has led to a broad range of applications from food preservatives to cosmetics[22]. Different studies have shown that rosmarinic acid has been reported to have some biological activities *in vitro* such as antiviral properties including anti-HIV-1[23], antibacterial, antioxidant, anti-carcinogenic ,[24-25] and anti-allergic activities [26,27]. In vivo studies have shown that rosmarinic acid (RA) exhibit anti-thrombotic, [28] This compound is also efficient against peroxidative damage to biomembranes [29]. Consequently, many products have been prepared from RA in pharmaceutical, cosmetic, and food industries. RA was found in many plants but usually rosemary plant is used as the major source.

In this work, we adopted fast and accurate chromatographic HPLC method using the (FLC, fast liquid chromatographic) column, 3um particle size and 5 cm length to simultaneously baseline separation of the main Rosmarinus compounds in less than 10 min , which can used for quality control and for follow up the effect of these components in various field of applications.

Preparation of authentic standards

Authentic standards were obtained from (Sigma) , methanol and propanol solvent were HPLC grade were from Merck .standard solution 25 µg/ml for each was prepared into 10 ml volumetric flask and dissolving in water: methanol: 2-propanol (each one contained 0.1% *O*-phosphoric acid) (60:30:10 , v/v) with the aid of ultrasonic bath.

Sample preparation

Fresh rosemary leaves were washed with deionized water and dried at 40 ◦C in a forced air circulation oven (Memmert), leaves were ground in a vertical hammer mill , then (1 gm) samples powder were accurately weighed into a 25-ml tube, and extracted with 25 ml of the same solvent system for preparing standard

solutions, during 30 min by ultrasonic bath. The resulting mixture was centrifuged at 4000 r/min for 5 min, and the supernatant transferred to a 100-ml volumetric flask. The residual solid was extracted for two more times with 25 ml of the same solvent mixture by ultrasonic, and centrifuged as above. The supernatants were combined, and diluted to 100 ml with the same solvent mixture. Then the extract were passed through 2.5 um disposable filter, and stored at 4°C for further analysis, then 50 ul were subjected into HPLC system according the optimum separation condition.

Instruments:

A Shimazdu 2010 A high performance liquid chromatograph system (Shimadzu, koyota, Japan) comprising vacuum degasser, quaternary pump, auto sampler , the eluted peaks were monitor by 20A SPD 2010 UV-VIS spectrophotometer at 285 nm. The analysis was performed on reversed phase (50x 4.6 mm I.D), 3µm particle size C-18 DB column, mobile phase were, water, solvent A: methanol, solvent B: 2-propanol , solvent C, the gradient mode was applied as shown in Table 1 (each one of mobile phase contained 0.1% *O*-phosphoric acid) , detection UV set at 285 nm, flow rate 1.2 ml/min , the temperature was maintained at 30 °C.

Table 1: Gradient time program for analysis of Rosmarinus constituents on reversed phase HPLC column

% of solvent A O-phosphoric acid in water	% of solvent B O-phosphoric acid in methanol	% of solvent C O-phosphoric acid in 2-propanol	Time(min)
10	20	70	0
15	20	65	3
20	60	20	2
20	60	20	5

The chromatographic peak of each constituent was confirmed by comparing the retention time related to the reference standard. Quantization was performed by comparing the peak area of sample with that of the standard under the same separation conditions.

RESULTS AND DISCUSSIONS

Although numerous of active compounds were reported in rosemary Extract some of them was traces, only , vanillic acid, caffeic acid, rosmarinic acid, naringin, hispidulin, cirsimaritin, carnosol, and carnosic acid were present in sufficient amount to be identified and quantified by adopted HPLC method. . 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 solvent and various extraction times. The best results were obtained by using the extraction procedure as in experiment section above.

By applied the optimum separation condition, the gradient program in (Table 1) were gave complete baseline separation for the standard of eight active constituents in less than 10 min , the same condition were applied for the extracted sample , as shown in typical chromatogram Fig (1) and table 2.

The quantification of rosemary compounds at 285 nm, which is wavelength at which all compounds were detected, 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 eight 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 naringin and carnosol (Table 2). Additionally, this method suitable when the plant distribution of these two main components was required in rosemary leaves extracts.

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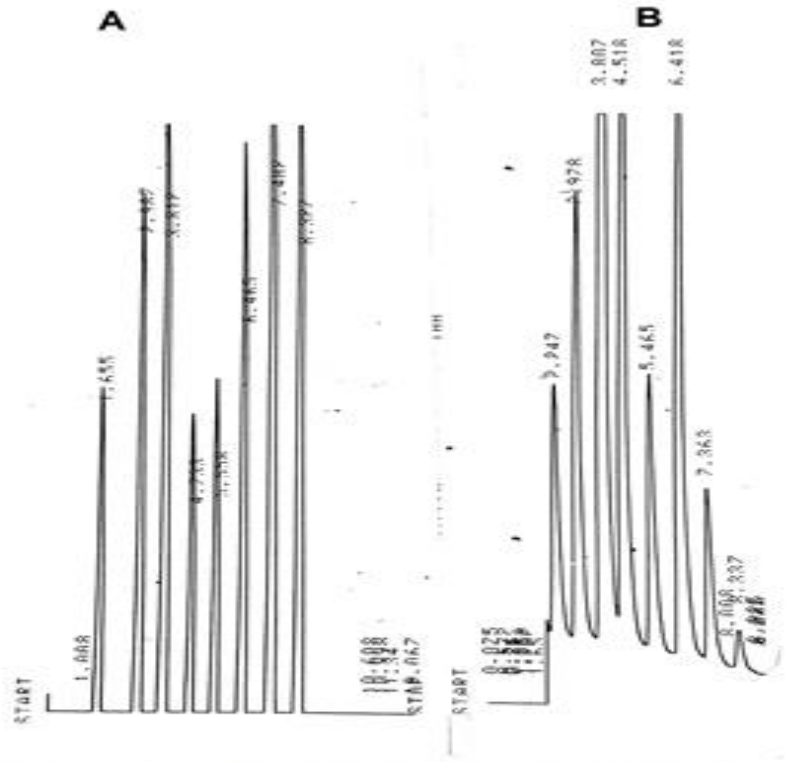

Fig (1): Reversed phase HPLC separation of antioxidant of Rosmarinus officinalis on the following optimum separation condition (as in text).

Chromatogram (A): Authentic Standard.

Chromatogram (B): Rosmarinus extracted sample.

Table 2; Identified compounds, retention time and their concentration levels in mg/gm fresh weight of extracted rosemary leaves. The data for n = 3 different determinations.

seq	Compounds	Retention time (min)	Concentration mg/gm
1	Caffeic acid	1.65	0.135 ±0.003
2	Cirsimaritin	2.98	0.257 ± 0.005
3	Carnosic acid	3.81	13.79 ± 0.598
4	Carnosol	4.73	0.775 ± 0.0354
5	Hispulin	5.55	0.097 ± 0.0758
6	Rosmarinic acid	6.46	3.927 ± 0.129
7	Naringin	7.40	0.848 ± 0.034
8	Vanllic acid	8.32	0.0210 ± 0.0042

The proposed analysis HPLC for simultaneous resolution of lipophillic and hydrophillic antioxidants is faster than the previously reported ones which is time consuming, lasting 30 or more minutes) [30] . Typical HPLC method by using the solid phase columns allow very good resolution in a short analysis time less than 10 minutes, were belong to choose suitable mobile phase to manage the separation rosmarinic acid which has low solubility in methanol . Therefore, some percent of methanol with water and propanol, allow complete separation of all constituent as shown in typical chromatogram (Fig 1)

CONCLUSIONS

The adopted chromatographic procedure employs a solid phase column with multi -solvent mobile phase permits a very fast separation of eight different constituents, specially, the more abundant, carnosic acid, carnosol and rosmarinic acid, in less than ten minutes.

This fast procedure shows a very good resolution and was developed to perform simultaneous determination of lipophilic and hydrophillic antioxidants present in the sample.

UV–vis measurements can be used to quantify these compounds in a fast and reproducible form. Samples of the same nature (extractions of dried leaves, (for example) can be measured, in order to have a similar profile for the unknown compounds. This method is appropriate for obtaining an accurate routine analysis of compounds content during harvest, or raw material quality control.

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