Determination of the Carnosic Acid Content in Wild and Cultivated *Rosmarinus officinalis*

P. J. Hidalgo,† J. L. Ubera,† M. T. Tena,‡ and M. Valcárcel*,‡

Department of Plant Biology and Ecology and Department of Analytical Chemistry, Faculty of Sciences, University of Córdoba, E-14004 Córdoba, Spain

The concentration of carnosic acid in a representative selection of leaves from wild and cultivated populations of *Rosmarinus officinalis* was determined by reversed-phase HPLC following extraction with supercritical carbon dioxide. Different sources of variability including season, genetics, leaf age, and growing origin (wild or cultivated) were considered. Variability in the carnosic acid content among rosemary leaves appears to be largely due to seasonal and environmental factors, in addition to their individual origin (genetics). The results reveal excellent correlation ($r = 0.93$) between the carnosic acid concentration and photoperiod. The results presented can be used to improve the selection of raw materials for the extraction of carnosic acid from rosemary.

Keywords: *Rosmarinus officinalis; carnosic acid; antioxidant; supercritical fluid extraction*

INTRODUCTION

Rosmarinus officinalis is a herbal spice that grows heavily in the Mediterranean basin. The distillation of its essential oils has been the traditional industrial use of rosemary. However, the extraction of food additives replacing synthetic food preservatives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) has lately gained much importance (Chen et al., 1992; Schwarz and Ternes, 1993; Lambelet et al., 1995). Carnosic acid is the major component in the phenolic diterpenoid fraction from *Rosmarinus officinalis* (Schwarz and Ternes, 1992; Okamura et al., 1994) and also that exhibiting the highest antioxidant activity (Aruoma et al., 1992; Cuvelier et al., 1994; Frankel et al., 1996a,b).

The determination of rosemary antioxidants usually entails liquid extraction with an organic solvent and uses a large amount of sample (Chen et al., 1992); also, it is scarcely reproducible and may result in partial degradation.

In fact, carnosic acid is prone to degradation (Okamura et al., 1994; Cuvelier et al., 1994; Ternes and Schwarz, 1995), particularly in polar solvents. In addition, its stability is strongly affected by pH (Frankel et al., 1996a,b), and its antioxidant activity is significantly greater in an acid medium.

Supercritical fluid extraction (SFE) methods are highly selective and provide clean extracts from small amounts of sample; also, they avoid the oxidation of carnosic acid (Tena et al., 1997).

The influence of environmental factors on lipid metabolism (Maffei et al., 1993) and biomass production in *R. officinalis* (Martínez-Fernández et al., 1994) was previously studied. However, the variability of phenol diterpenoid contents in *R. officinalis* introduced by environmental and genetic factors remains unknown. The aim of this study was to determine the factors that influence the carnosic acid content in *R. officinalis* leaves. Thus, the concentration of carnosic acid was determined in various individuals of *R. officinalis* of two different ages grown under two different types of conditions; also, the carnosic acid content in the rosemary leaves was monitored over a 15-month period. The results provide revealing information on the synthesis and accumulation of phenolic diterpenoids and are highly useful with a view to maximizing the use of rosemary.

MATERIALS AND METHODS

Apparatus. The experiments conducted to validate the method were previously published elsewhere (Tena et al., 1997). Once the optimal SFE conditions were established, three successive extractions of the same rosemary sample (20 mg) were carried out to ensure completeness of the SFE. The amounts of carnosic acid found in the first, second, and third extractions were 95, 4, and 1%, repectively, of the total amount extracted. The SFE was also compared with liquid solvent sonication. Among the liquid solvents studied, only exhaustive extraction using acetone provided comparable results (73% recovery relative to SC−CO_2 extraction).

Rosemary leaves were extracted with supercritical pure carbon dioxide at 383 bar, at 120 °C, and at 4 mL/min flow rate (liquid) on a Hewlett-Packard 7689A supercritical fluid extractor furnished with a 7-mL extraction vessel, a variable restrictor, and a solid-phase trap packed with octadecylsilica (ODS). No modifier was used, and acetone was used as rinsing solvent. A Hewlett-Packard 1050 liquid chromatograph equipped with a 20-μL loop injector, an Ultrabase C_{18} (250 × 4.6 mm, 5 μm) column, and an HP 1040A diode array detector were used to separate and detect carnosic acid in the extracts. The flow rate of the mobile phase was 1 mL/min. An acetonitrile/10 mM acetic acid solution gradient from 70:30 (for the first 8 min) to 100% acetonitrile in 5 min was used. The experimental procedures were described elsewhere (Tena et al., 1997). The precision of the overall method (SFE + HPLC), as percent relative standard deviation ($n = 7$), was 3.6%.

Chemicals and Plant Material. Carnosic acid was complimentarily supplied by Prof. N. Okamura (Fukuyama University, Fukuyama, Japan). The plant material studied was obtained from wild populations in the province of Córdoba (Andalucía, southern Spain) and from experimental cultures derived from them. *R. officinalis* specimens were collected

† Department of Plant Biology and Ecology.
‡ Department of Analytical Chemistry.

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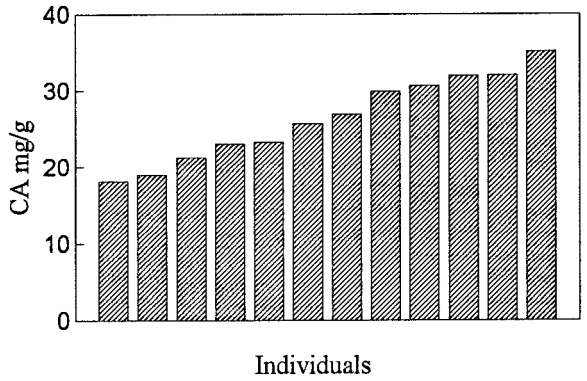

Figure 1. Distribution of carnosic acid content in leaves of individuals from a homogeneous wild population.

between February 1996 and May 1997. Fresh rosemary leaves were dried at 50 °C, ground, and sieved (≤500 μm). All carnosic acid contents reported are referred to dry rosemary weight and are the average of two replicates.

Sampling Strategies. The following guidelines were observed in the sampling procedure:

(1) For the study of within-population variability, 12 individuals were selected from a homogeneous wild population. All individuals were under identical environmental conditions and sampled on the same day (April 8, 1996) to exclude other sources of variability such as environmental factors and collection period.

(2) To study the influence of plant age, the carnosic acid content in actively growing young leaves and in mature leaves of four wild individuals was determined. The sampling was carried out in March 1996, when vegetative growth began.

(3) Seasonal variability was estimated by monitoring the carnosic acid content in leaves from an individual over a 15-month period. The amount of leaves sampled was as small as possible; also, plants were collected at long intervals (~1 month) to ensure representativeness and to avoid disturbing plant development.

(4) The influence of the cultivation conditions was established by comparing the carnosic acid contents in three different individuals and those in plants grown from their pegs. The latter plants were cultivated under optimal growing conditions for 2 years over a calcic rodoxeralf soil, irrigated at rates of 8 L/m^2 per week in spring and autumn and 16 L/m^2 per week in summer, in addition of the rainfall. No fertilizers were used. Both series of rosemary leaves were sampled on the same date to avoid any seasonal differences in the carnosic acid content.

RESULTS AND DISCUSSION

Within-Population Variability. Figure 1 shows the differences in carnosic acid content among individuals of the same population. The carnosic acid content in leaves of 12 *R. officinalis* individuals was found to be highly variable (from 18.2 to 35.1 mg/g). Differences of up to 50% among individuals of the same population are thus to be expected. No specific concentration value was particularly frequent.

The *R. officinalis* individuals studied differed widely in size and shape of both plant and leaves, as well as in leaf color depth and brightness and flower color, among others. However, we found no correlation between plant appearance and carnosic acid content. The variability seems to be of genetic origin alone, even though other factors influencing the carnosic acid content (e.g., microenvironmental factors, plant age) cannot be excluded. High individual variability of a markedly random nature was previously reported in a study on biomass production (Martínez-Fernández et al., 1994).

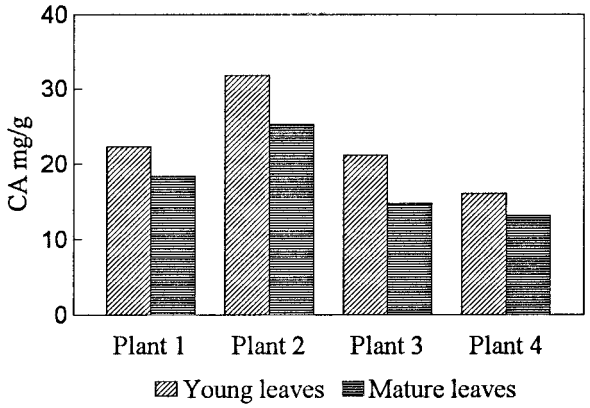

Figure 2. Influence of leaf age on the carnosic acid content in four different individuals.

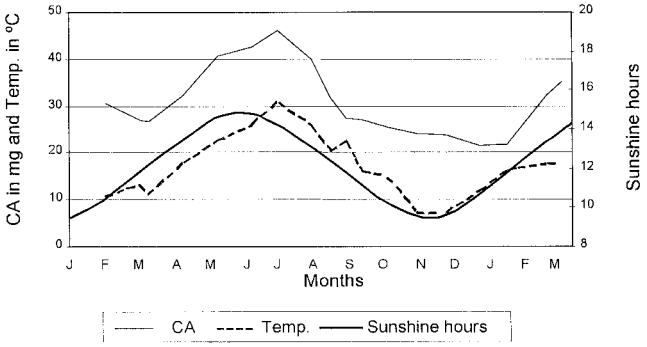

Figure 3. Changes in carnosic acid content in rosemary leaves over a 15-month period. The graph also shows the theoretical day length (sunshine hours) and the temperature (5 day floating mean) for the period studied.

Carnosic Acid Content in Young and Mature Leaves. Figure 2 shows carnosic acid contents in young and mature leaves from four different individuals. The contents in mature leaves were all 21 ± 5% smaller than those in young leaves, which is consistent with the following:

(1) Protective compounds such as antioxidants are essential at the early growth stages, where young leaves are more vulnerable.

(2) Antioxidants are located predominantly in leaves and are almost absent from woody parts such as stems (Okamura et al., 1994). The living/lifeless tissue ratio in newly formed leaves is higher than in old leaves.

(3) Young leaves have a far more active metabolism, so they require higher concentrations of the essential compounds needed for growing.

Seasonal Variability of the Carnosic Acid Content. Figure 3 shows changes in the carnosic acid content in leaves of a *R. officinalis* individual over a 15-month period. The carnosic acid content increased gradually during the spring, peaked in the summer months (46.15 mg/g on July 26, 1996), and then dropped abruptly until the end of September, after which time it continued to fall, more slowly, to a minimum at 21.4 mg/g on February 11, 1996.

Changes in the carnosic acid content during the year seem to be related to the weather conditions to which the plant is exposed. In addition to the carnosic acid content, Figure 3 shows the distribution of the theoretical number of sunshine hours and the temperature (5-day floating mean) for the period studied, which influence the content. Both parameters were found to be correlated with the carnosic acid content by 82 and 81%,

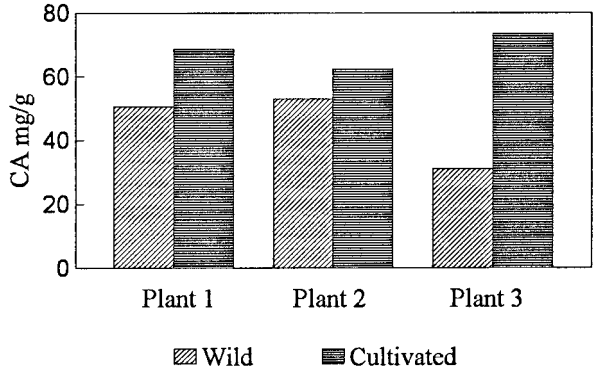

Figure 4. Effect of the growing conditions (wild or cultivated) on the carnosic acid content in rosemary leaves.

respectively (percent correlation coefficient of the linear regression by least-squares method). If the day length curve is delayed 19 days, then correlation with photoperiod improves up to a maximum of 93% but those with temperature decrease to 71%. Photoperiod and temperature are the primary environmental controls over time of flowering and maturity, cultivar adaptation, and yield (Wallace et al., 1993). The significance of day length to seasonal variability in the carnosic acid content is apparent from the excellent correlation found; the effect is delayed 19 days. The correlation with temperature was lower, with a maximal correlation for the same day of sampling. However, the influence on carnosic acid content by other seasonal factors such as rainfall (data not shown) should be underrated, as they would affect a longer and irregular period.

Vegetative growth in rosemary peaks in spring (Martínez-Fernández et al., 1994) and is strongly dependent on soil moisture but scarcely correlated to rainfall and temperature. The largest numbers of fallen leaves are observed in August/September (Martínez-Fernández et al., 1994). Leaf falling starts at the beginning of an abrupt drop in the carnosic acid content. The plant material sampled in summer was likely to contain a higher proportion of old leaves and thus less carnosic acid, as previously established.

The increased carnosic acid levels in rosemary leaves during the period of maximum sunshine confirm the protective role ascribed to this antioxidant. This type of phenolic diterpene has been shown to effectively protect biological systems from oxidative stress (Haraguchi et al., 1995). Thus, it acts as a potent inhibitor for peroxidation in the highly polyunsaturated fatty acids present in biological membranes. Of all antioxidative diterpenoids in *R. officinalis*, carnosic acid is the most effective inhibitor for production of superoxide anion by the xanthine oxidase system; also, it surpasses flavonoids, well-known as scavengers of superoxide anions, in this respect (Haraguchi et al., 1995). The lowest carnosic acid contents occur in the months of least sunshine and start to rise at the end of April. On the other hand, epicuticular fatty acids accumulate during winter (Maffei et al., 1993), which appears to be an adaptative metabolic response to low temperatures.

Influence of the Growing Conditions. As is apparent from Figure 4, cultivated plants exhibited higher carnosic acid contents than their wild precursors in all cases. The wild individuals had widely variable contents of the acid (from 31.1 to 53.1 mg/g), whereas the cultivated individuals had more uniform contents (between 68.8 and 73.5 mg/g). Individuals placed under cultivation conditions for 2 years grew uniformly and reached a large size, in contrast to the sparse, uneven growth of the wild individuals. The highest carnosic acid content (73.5 mg/g) was found in a cultivated rosemary plant (Figure 4, plant 3), despite the very low content in its wild precursor (31.1 mg/g).

The chromatograms for the extract from each cultivated individual and that from its wild precursor exhibit peaks at the same retention times but with different areas; this indicates that, notwithstanding the concentration differences, the spectrum of compounds is characteristic for each individual.

On the basis of these results, optimizing the growing conditions may be one way of boosting production of carnosic acid by *R. officinalis*. Irrigation seems to increase carnosic acid contents and decrease differences among wild individuals.

Conclusions. Variability in the carnosic acid content in *R. officinalis* leaves was studied. The content in leaves of wild rosemary individuals was found to be highly variable. Mature leaves contain smaller amounts of carnosic acid than young leaves. The carnosic acid content peaks in summer and is greater in rosemary individuals grown under cultivation conditions. The results are highly promising with a view to the industrial production of antioxidants from rosemary, which is in its early stages.

Finally, the use of a rapid, automated new methodology such as SFE with carbon dioxide for the extraction of carnosic acid affords a higher precision and accuracy than those achieved with labor-intensive conventional liquid extraction methods. In addition, SFE uses substantially reduced amounts of sample.

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