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Fast high performance liquid chromatography and ultraviolet–visible quantification of principal phenolic antioxidants in fresh rosemary

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Abstract

An improved HPLC method is reported for the determination of rosemary's principal phenolic antioxidants, rosmarinic and carnosic acids, providing a fast and simultaneous determination for both of them by using a solid phase column. The analysis was performed with fresh methanolic extractions of *Rosmarinus officinalis*. To quantify the amount of antioxidants in a fast and reproducible way by means of UV–vis absorption measurements, a spectrophotometric multi-wavelength calibration curve was constructed based on the antioxidant contents obtained with the recently developed HPLC method. This UV–vis methodology can be extended to the determination of other compounds and herbs if the restrictions mentioned in the text are respected.

Keywords: *Rosmarinus officinalis*; Carnosic acid; Rosmarinic acid; Carnosol; Phenolic antioxidants; HPLC analysis

1. Introduction

An antioxidant may be roughly defined as "any substance that when present at low concentrations, lower than the oxidizable compound to be protected, significantly delays or inhibits its oxidation". 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, there is an increasing interest in finding naturally occurring antioxidants for food and medicinal applications. The extraction of natural substances to replace synthetic food preservatives has become increasingly more important [3–10]. 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*) [11]; thymol from thyme (*Thymus vulgaris*) [12]; carnosic acid (CA) from rosemary (*Rosmarinus officinalis*) [13]; rosmarinic acid (RA) from oregano, rosemary, sage (*Salvia officinalis*) [14,15] or lemon balm (*Melissa officinalis*) [16]. Other phenolic antioxidants of commercial interest are those from grape seeds, pine bark, and some fruit fractions rich in gallic acid. For most of these, there are no analytical methodologies able to resolve two or more antioxidants or active principles simultaneously in a fast and precise way. When production is based on these types of herbal materials, the lack of such a methodology could be critical for the development of product quality control and procedures.

Currently, the HPLC determination of natural compounds is time consuming, requires a large sample and entails liquid extraction with an organic solvent [3]. We present a fast chromatographic methodology and a clean and inexpensive spectrophotometric analytical model intended to analyze simultaneously different phenolic antioxidants present in *R. officinalis*.

The reported analytical procedures were applied to quantify rosemary phenolic antioxidants. A lengthy study of the seasonal antioxidant behaviour profile of five accessions of rosemary was made. A dozen seed-raised accessions (individual by individual) and more than a hundred genetically uniform accessions are being studied according to their antioxidant content. The large number of analyses necessitated the development of a fast, reliable and inexpensive quantification procedure. Among

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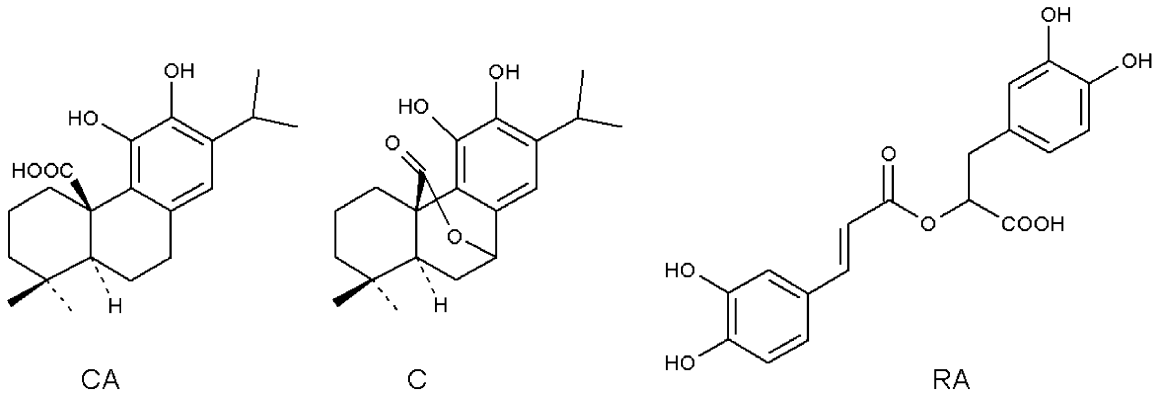

Fig. 1. Chemical structures of three of the phenolic antioxidants present in rosemary methanolic extracts: carnosic acid, CA; carnosol, C; and rosmarinic acid, RA.

the existing techniques, direct spectroscopies are the preferred ones, with UV–vis methodologies being the ones that are most used because of their simplicity and wide availability [17]. NIR methodologies have also been applied in principal component analysis, PCA, authentication and quantification [18], but they require specialized and expensive equipment and software. In addition, the construction of calibration curves requires a very large number of experimental measurements.

Carnosic acid is the major component in the phenolic diterpenoid fraction from *R. officinalis* leaves, with the highest antioxidant activity [13,15,19]. The other abietane diterpene present in rosemary extracts is carnosol (C). In aged samples and extracts, increasing amounts of this compound are observed, which is in accordance with the carnosic acid degradation profile proposed [16]. The other active antioxidant present in this herb is hydro-soluble rosmarinic acid. Fig. 1 shows the chemical structures of the previously mentioned antioxidants.

2. Experimental

2.1. Materials

Rosmarinic acid and carnosic acid, used as standards, were obtained from Addipharma and Sigma, respectively. The water used was Milli-Q quality, methanol and acetonitrile (MeCN) were from Merck and HPLC grade. Inorganic reagents from Merck were PA quality.

Plant accessions were kindly borrowed from the special collection used by BOTANE Ltd. to establish its crops and were collected throughout Chile and other countries. If possible, accessions were clonally propagated in order to maintain genetic uniformity.

Each accession was propagated and established in a greenhouse and then transplanted to square-shaped blocks of 36 individuals. Accessions were planted in November 2001 at Illapel, in the north central Chile, about 250 Km north of Santiago (31°43′ S; 71°07′W; 391 m.a.s.l.). Sampling was performed in a random pattern in the square shaped blocks and on freshly grown material. In order to have more varied sources of antioxidant content sampling was made in all seasons. The accessions propagated from seeds were sampled individually in order to select the most promising ones.

2.2. Sample preparation

Fresh rosemary samples were dried at 40 °C in a forced air circulation oven (Memmert ULM 500), leaves were manually separated and ground in a vertical hammer mill (Peruzzo Milly model 35.010) at 12,000 rpm and 0.8 mm mesh. Sample humidity was determined employing a Sartorius MA30 infrared system. Fifty milligrams of the ground sample were extracted during 5 min with 25 ml of methanol or methanol:water in an Elma LC30H ultrasonic bath, avoiding warming. Before the analysis, samples were centrifuged for 3 min at 3,500 rpm (Damon/IEC Division, model IEC HN-SII).

2.3. Instrumentals

The instrumental setup used for analytical HPLC was conducted with a Merck-HITACHI LaChrom system, with a L-7100 pump combined with a 10 mm pre-column and a 100–4.6 mm Merck Chromolith Performance RP-18e, a Merck-HITACHI photodiode array detector DAD L-7450. The system was controlled with HPLC D-7000 software with a D-7000 data interface.

For the spectral analysis, a Unicam UV500 UV–vis spectrophotometer controlled with Software Vision 32-bit Version 1.10 was used. From the absorption spectra of sample solutions, absorbances at 230, 270, 280 and 330 nm were obtained.

2.4. Chromatographic conditions

A binary MeCN-H_2O acidified gradient was used for elution. Two different procedures were developed. Method I, for simultaneous resolution of the three compounds of interest (CA, C, RA), the solvents A and B were MeCN–H_2O–H_3PO_4 (65.1%:34.9%:0.02%) and MeCN–H_2O–H_3PO_4 (22%:78%: 0.25%), respectively. At a flow of 1.5 ml/min, the eluent consisted of 100% B during the initial 2 min, then, the percent of solvent A was increased to 100% at 2.1 min and remained at this percentage during the next 6 min. At 8.1 min the percent of solvent B was again increased to 100%, where it remained the last 2 min of run time. With this method the retention times were, for RA t_r = 2.7 min, for C t_r = 5.7 min and for CA t_r = 6.6 min. This method requires sample extraction with methanol:water (2:1),

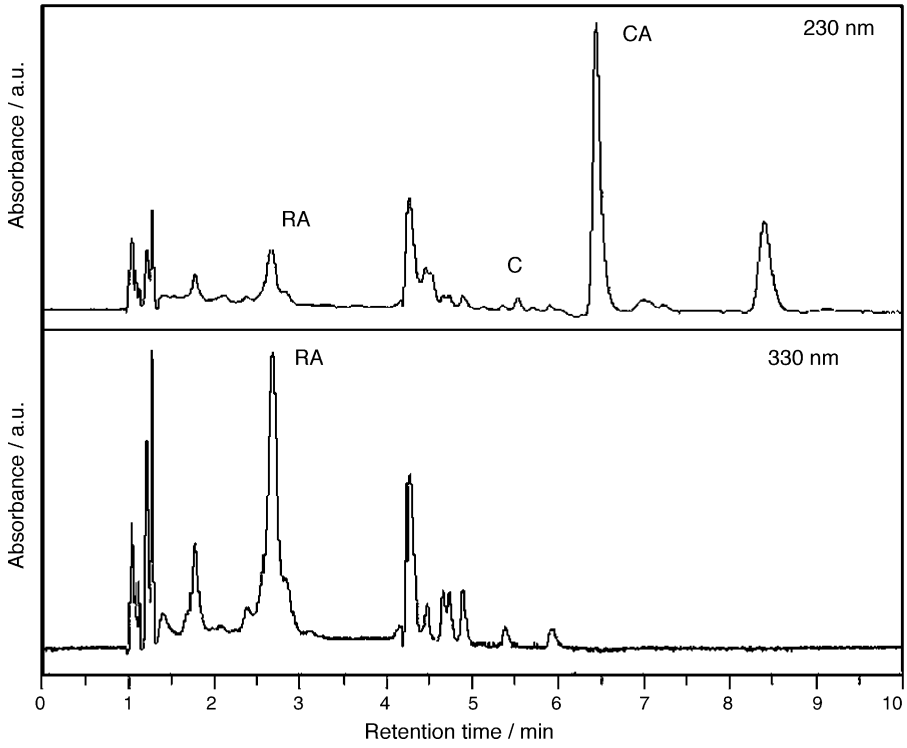

Fig. 2. Chromatograms obtained with Method I at 230 and 330 nm.

in order to extract all lipo-soluble and hydro-soluble antioxidants. The peak labelled as RA mainly consists of rosmarinic acid, however low concentrations of some of its derivatives are also present. Consequently, several shoulders can be seen around peak [20]. Results obtained with this method are shown in Fig. 2. Method II, this chromatographic procedure is isocratic with solvent A as eluent during 6 min. The retention times were, for C t_r = 1.8 min and for CA t_r = 2.5 min. With this procedure, hydro-soluble compounds are not resolved, so sample extraction was simply performed with methanol. A typical chromatogram obtained with this method is shown in Fig. 3.

The detection wavelengths selected to quantify CA and RA were 230 and 330 nm, respectively, in order to avoid mobile phase absorption. (The selection corresponds to a shoulder of CA absorption and a maximum of RA, as can be seen in Fig. 4.)

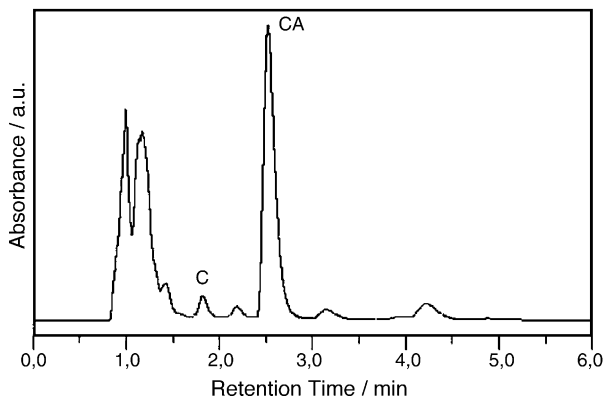

Fig. 3. Chromatogram obtained with Method II at 230 nm.

2.5. Statistical analysis

Equation coefficients and statistical parameters of performed correlations were obtained by multilinear correlation analysis with STAT VIEW 5.0 (SAS Institute Inc.). Results agree favourably with the *t*-statistic of descriptors.

3. Results and discussion

The amount of the antioxidants studied (CA and RA) present in fresh rosemary extracts of fresh leaves was determined by using the chromatographic methodology I, described above. As mentioned, Fig. 2 shows typical chromatograms obtained with

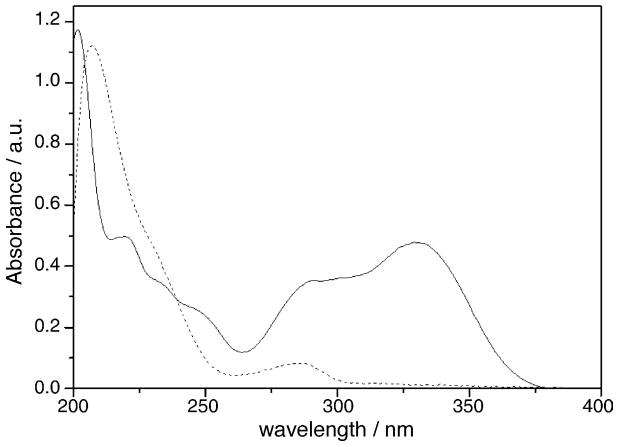

Fig. 4. Absorption spectra of carnosic acid, CA (dashed line) and rosmarinic acid, RA (solid line).

Table 1
Correlation equation of carnosic acid content using dry basis weight of leaves with the absorbance at three different wavelengths, statistical descriptors for parameters and fitting are included

CA content (%) = $C_0 + aA_{230} + bA_{270} + cA_{330}$				
	C_0	a	b	c
	(0.19 ± 0.51)	(3.86 ± 0.28)	$-(4.70 \pm 0.95)$	$-(1.37 \pm 0.46)$
t-statistic	0.374	13.817	−4.945	−0.492
P (two-tailed)	0.7121	<0.0001	<0.0001	0.0067
VIF		4.31	15.87	8.85
$N = 27$	$R = 0.964$	SD = 0.384		$F = 99.65$

this procedure at 230 and 330 nm. As can be seen the Merck solid phase columns RP 18 allow very good resolution in a short analysis time. The proposed analysis for simultaneous resolution of lipophillic and hydrophillic antioxidants (Method I) is faster than the previously reported ones (this kind of analysis used to be time consuming, lasting 30 or more minutes) [14,21–23]. Typical HPLC methodologies reported for quantification of abietane diterpenoids (determination of only carnosic acid and carnosol) require at least 20 min [24]. Method II allows the resolution of CA and C in only 6 min of analysis. As mentioned in Section 2, rosmarinic acid is present in small quantities because of its low solubility in the extraction solvent, methanol, and it also appears in the front of elution, so it is not resolved. In the presence of water, CA shows low stability, so pure methanol was used to perform extraction (when some percent of water is present for extraction, samples must be analyzed quickly in order to avoid or reduce conversion of CA to C). Studies of CA solutions stability are reported by Thorsen and Hildebrandt [24]. The HPLC methodologies that were developed show a percentage coefficient of variation in the order of 3.5%.

The values obtained for the CA content, show high variability between the different accessions, with percentages ranging from 2% to 8% on dry basis weight. This result indicates that the ability or capability of CA generation by rosemary is particularly dependent on its genetic origin, considering homogeneous seasonal, geographical and agronomic parameters, conclusions previously reported by other authors [21,25]. At this point, it must be emphasized that carnosol is a compound usually measured in rosemary samples, but it mostly occurs with the degradation of carnosic acid. Its presence in high concentrations indicates either sample aging or bad manipulation, which is why only small amounts of carnosol are expected in fresh extracts of fresh samples. Contents of carnosol in our samples were usually under the detection limit and consequently no spectroscopic curves were made.

The absorbance spectrum of the different samples was recorded, and four wavelengths (230, 270, 280 and 330 nm) were used to perform the multiparametric analysis. The wavelength selection criteria was to use intense absorption bands, preferably maxima, of the analyzed compounds and possible interferents. Wavelengths corresponding to maxima under 215 nm were discarded considering the proximity of solvents cutoff and overpopulation of the region. Several procedures in multivariate UV analysis have been reported, indicating different options for wavelength selection in order to improve quantification [17,26]. Considering the fact that our samples contain a large number of unknown components, we decided not to employ the procedures associated with multivariate analysis. The samples' spectra always displayed a similar shape with differences in the relative magnitude of absorbance at each wavelength.

The general equation employed has the form shown in Eq. (1). The antioxidant content determined by HPLC was the dependent variable of the multiparametric equation. The independent variables (absorption at each wavelength) were normalized according to dry mass and sample dilution.

$$\text{Content}\,(\%) = C_0 + aA_{230} + bA_{270} + cA_{280} + dA_{330} \qquad (1)$$

where the parameter C_0 is the intercept and parameters a, b, c and d correspond to the input at each wavelength to the content of the compound to be quantified.

Statistical analysis for correlation equation performed for CA content rejects the absorption at 280 nm, meaning that only three of the four independent variables are significant. The values obtained for each wavelength, its statistical descriptors and the statistical parameters for the whole treatment are shown in Table 1. The intercept C_0 takes a value near zero, corresponding to the absence of CA when the absorption is null. The parameter associated with the absorbance at 230 nm indicates an important input of CA at this wavelength. Clearly, the absorptions at 270 and 330 with negative contribution correspond to the presence of compounds other than CA. The absorption at 270 nm is probably due to the presence of chlorophylls or essential oils and the one at 330 nm corresponds to the presence of RA. The standard error for each parameter is somewhat high, but good enough to perform a fast and adequate quantification. t-statistic and P are statistically satisfactory parameters. The high VIF values, over 3.0, indicate some extent of CA absorption at all wavelengths. The overall statistical quality parameters for fitting equation are good, the R-value is 0.964, SD is small (0.384) and F is very high (99.649).

The quality of the fit with experimental data can be seen in Fig. 5, where contents calculated with the multiparametric equation that was obtained are plotted against experimentally measured contents.

For the antioxidant RA, the multiparametric study rejects the parameters associated with two wavelengths, leaving only the absorbances obtained at 270 and 330 nm in the correlation. The resulting equation is shown in Table 2. The intercept C_0 has a

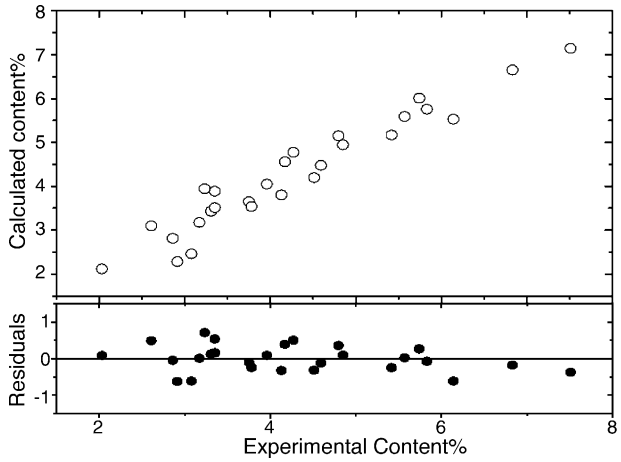

Fig. 5. Plot of the multi-wavelength fit (three wavelengths equation) against the experimental values of carnosic acid content, including the residuals plot.

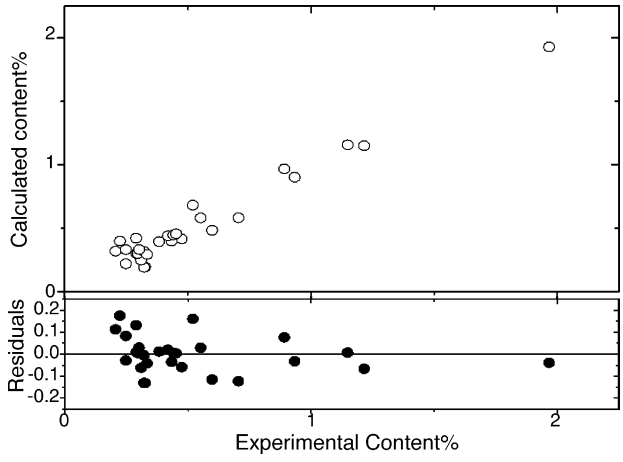

Fig. 6. Plot of the multi-wavelength fit (two wavelengths equation) against the experimental values of rosmarinic acid content, including the residuals plot.

value near zero, while the negative parameter associated with the absorbance at 270 nm is due to the presence of chlorophylls. The parameter corresponding to the absorbance at 330 nm is the RA contribution to the absorbance. This correlation shows lower standard errors and both *t*-statistic and *P* are better than for CA correlation. The independent parameters still show high VIF values, due to the reasons stated above. The overall statistical quality parameters are good, the *R*-value in this case is 0.978, SD is very small (0.085) and *F* is very high (268.96), indicating a high-quality correlation equation.

Fig. 6 shows the experimental results obtained when employing this fitting equation.

The measured percent coefficient of variation for spectroscopic procedures is 1.1% for CA, and 2.5% for RA, being both lower than the value determined for HPLC methodology.

Attempts to include samples of commercial extracts and dry rosemary samples together with our own extracts in the same correlation were always unfruitful. The presence of some absorbing compounds or the absence of others yields a different distribution of absorbing species, making the multiparametric equation rather inadequate. If raw material and extraction procedure are maintained, the construction of a multiparametric calibration curve could probably be extended to any group of natural compounds, providing an interesting tool to perform fast and reliable quantifications.

Table 2
Correlation equation of rosmarinic acid content using dry basis weight of leaves with the absorbance at two different wavelengths, statistical descriptors for parameters and fitting are included

RA content (%) = $C_0 + aA_{270} + bA_{330}$			
	C_0	a	b
	(0.11 ± 0.12)	$-(0.84 \pm 0.15)$	(1.28 ± 0.09)
t-statistic	0.915	−5.685	13.717
P (two-tailed)	0.3691	<0.0001	<0.0001
VIF		7.04	7.04
$N = 28$	$R = 0.978$	SD = 0.085	$F = 268.96$

4. Conclusions

The reported chromatographic procedure 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 multi-wavelength calibration curve can be constructed with a subset of samples, if the content of the compounds of interest is known. UV–vis measurements can then be used to quantify these compounds 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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