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# Flavonoid Distribution during the Development of Leaves, Flowers, Stems, and Roots of *Rosmarinus officinalis*. Postulation of a Biosynthetic Pathway

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The distribution of seven flavonoids, eriocitrin, luteolin 3′-*O*-*β*-D-glucuronide, hesperidin, diosmin, isoscutellarein 7-*O*-glucoside, hispidulin 7-*O*-glucoside, and genkwanin, has been studied in *Rosmarinus officinalis* leaves, flowers, stems, and roots during plant growth. The maximum level reached by luteolin 3′-*O*-*β*-D-glucuronide in leaves during June−August suggests the existence of a delay between the activation of the enzymes involved in the flavanone and flavone biosynthesis. The presence of hesperidin and diosmin in the vascular system is significant, and hesperidin shows even higher levels than the phenolic diterpenes and rosmarinic acid. The distribution of flavonoids observed in *R. officinalis* suggests a functional and structural relationship between phytoregulators and flavonoids, where flavonoids would be "protectors" of the activity of phytoregulators. A hypothesis for the general pathway of biosynthesis of these compounds in plants of the family Labiatae is proposed.

**KEYWORDS:** ***Rosmarinus officinalis*; flavonoids; eriocitrin; luteolin 3′-*O*-*β*-D-glucuronide; hesperidin; diosmin; isoscutellarein 7-*O*-glucoside; hispidulin 7-*O*-glucoside; genkwanin**

## INTRODUCTION

*Rosmarinus officinalis* is well-known for its antioxidative properties, which mainly depend on the presence of rosmarinic acid and phenolic diterpenes, such as carnosic acid, carnosol, rosmanol, and epirosmanol, among others. Many studies have dealt with the characterization and identification of these polyphenolic compounds present in rosemary (*1*−*6*). Rosemary extracts containing rosmarinic acid and phenolic diterpenes are widely used in the food industry, where they are used as alternatives to synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA); furthermore, they exhibit synergy with other natural antioxidants such as ascorbyl palmitate and α- and *δ*-tocopherol mixtures.

Moreover, the applications for which rosemary extracts are used are not only due to the above-mentioned compounds, but also the flavonoids present in the plant increase the therapeutic properties of rosemary, especially in the field of cardiovascular diseases, where other plants, such as *Citrus* species have been seen to be of use (*7*). If the antioxidant activity of the main phenolic diterpenes present in rosemary could be combined with some of the flavonoids present, the potential of these extracts would increase enormously.

However, very few studies have been published on the qualitative and quantitative presence of flavonoids in *R. officinalis* and other Labiatae plants (*8*−*15*). In this study we investigated this presence by analyzing the quantitative distribution of flavonoids during the development of the different plant organs in rosemary and the relationship between their biosynthesis and transport processes.

## MATERIALS AND METHODS

**Plant Materials.** Rosemary leaves, flowers, stems, and roots were obtained from 3-month-old *R. officinalis* plants (initiation cycle) grown in greenhouses at the University of Murcia. The growing conditions were selected according to the procedure of Munné-Bosch (*16*). The plant materials were collected between February 2001 and February 2002 and immediately dried at 40 °C, obtaining between 3 and 5 g of each dried organ. Then, the material was ground to a homogeneous powder (particle size <0.5 mm in diameter) for extraction and analysis.

**Materials and Reagents.** Eriocitrin, hesperidin, diosmin, and genkwanin were obtained from Extrasynthèse (Genay, France). Luteolin 3′-*O*-*β*-D-glucuronide, isoscutellarein 7-*O*-glucoside, and hispidulin 7-*O*-

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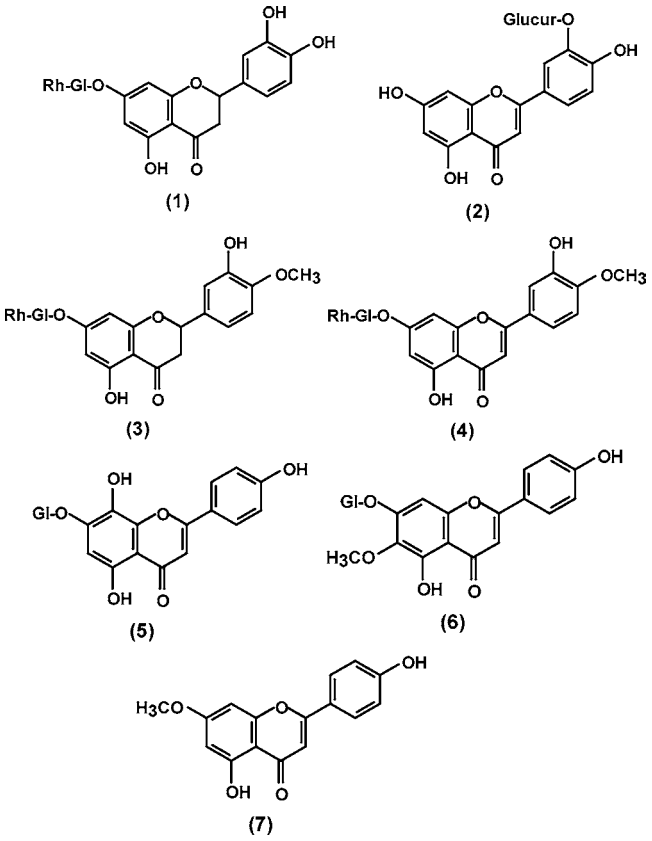


**Figure 1.** Chemical structures of the compounds studied: eriocitrin (**1**); luteolin 3′-*O*-*β*-D-glucuronide (**2**); hesperidin (**3**); diosmin (**4**); isoscutellarein 7-*O*-glucoside (**5**); hispidulin 7-*O*-glucoside (**6**); genkwanin (**7**). Rh, rhamnose; Gl, glucose.

**Table 1.** Experimental Conditions for Mass Detection

| parameter | value |
|---|---|
| capillary (kV) | 3.5 |
| cone (V) | 30–60 |
| extractor (V) | 2 |
| RF Lens (V) | 0 |
| source temperature (°C) | 80 |
| desolvation gas | nitrogen |
| desolvation gas flow (L/h) | 350 |
| desolvation temperature (°C) | 200 |
| cone gas flow (L/h) | 150 |

glucoside were obtained by HPLC semipreparative methods from rosemary extracts.

HPLC grade methanol, acetonitrile, acetic acid, dimethyl sulfoxide (DMSO), and water were obtained from Merck (Darmstadt, Germany).

**Extraction of Flavonoids from Plant Materials.** The flavonoids were extracted for analytical chromatography from previously dried plant materials using DMSO in the ratio of 20 mg/mL. The solvent was selected by previous findings on flavonoid extraction (*17*). The extraction was done at 25 °C during 20 min in a stirred flask. The measurements were performed on six plants, and the values obtained at each age were used to express the distribution of flavonoids in leaves, flowers, stems, and roots (percent dry weight). All of the solutions were filtered through a 0.45 $\mu$m nylon membrane.

**Chromatographic Analysis.** Three HPLC methods were used for the location, identification, and quantification of the flavonoids in plant materials, combining and modifying other previously described methods (*2*, *15*, *18*, *19*). The HPLC equipment used was a Hewlett-Packard HP 1100 equipped with a diode array detector. The stationary phase was a 250 × 4 mm i.d., 5 $\mu$m, $C_{18}$ LiChrospher 100 analytical column (Merck) thermostated at 30 °C. The flow rate was 1 mL/min, and elution was monitored at 280 nm.

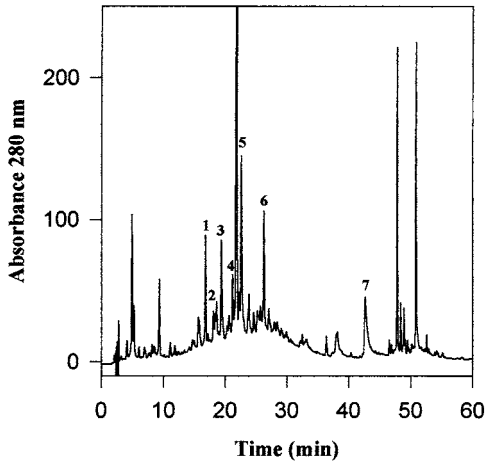


**Figure 2.** Characteristic chromatogram of a DMSO extract from *R. officinalis* leaves. Peak numbering is as for **Figure 1**. Peak with $t_R$ 22 min is rosmarinic acid. Peaks with $t_R$ between 47.5 and 54.0 min are phenolic diterpenes.

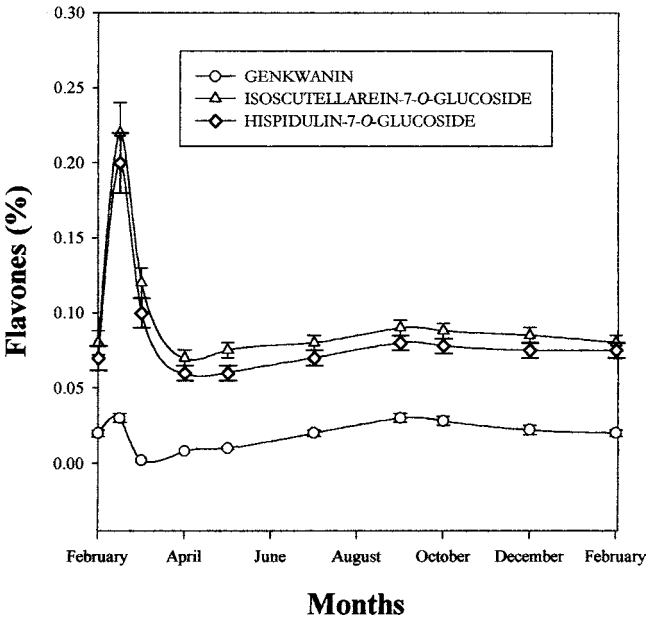


**Figure 3.** Changes in the mean total content (percent dry weight) of isoscutellarein 7-*O*-glucoside, hispidulin 7-*O*-glucoside, and genkwanin according to age in *R. officinalis* leaves (2001–2002 season).

The mobile phases for method 1 were water with 2.5% acetic acid (A) and acetonitrile (B); 95% A was maintained for 20 min and then changed to 50% A in 20 min, which was then changed to 20% A in 10 min and reequilibrated in 10 min to the initial composition.

The mobile phases for method 2 were water with 1% acetic acid (A) and methanol (B). An isocratic step of 50% B during 5 min was run, followed by a linear gradient to 100% B in 25 min. This composition was maintained for 10 min, and then the system was reequilibrated to the initial composition in 5 min.

The mobile phases for method 3 (an isocratic run) were water with 2.5% acetic acid (A) and acetonitrile (B) using 83% A and 17% B.

Phenolic compounds were located and identified by comparison of their retention times with the corresponding standards and by their UV spectra obtained with the diode array detector.

**Isolation and Identification of Flavonoids.** For the isolation of the main flavonoids (**1**, eriocitrin; **2**, luteolin 3′-*O*-*β*-D-glucuronide; **3**, hesperidin; **4**, diosmin; **5**, isoscutellarein 7-*O*-glucoside; **6**, hispidulin 7-*O*-glucoside; **7**, genkwanin) shown in **Figure 1**, 50 g of leaves was extracted with DMSO in the ratio of 200 mg/mL. The solutions were filtered through a 0.45 $\mu$m nylon membrane, and a 250 × 10 mm i.d., 5 $\mu$m, $C_{18}$ semipreparative column was used with a flow rate of 3 mL/

**Table 2.** Retention Time ($t_R$) (Minutes), UV Absorbance Maximum, and Molecular Ion of the Flavonoids Present in *R. officinalis*

| flavonoid | method 1 | method 2 | method 3 | UV absorbance max (nm) | molecular ion peak (*m*/*z*)[a] |
|---|---|---|---|---|---|
| eriocitrin | 16.80 | 2.80 | 7.80 | 285 | 597 $[M-1]^-$ |
| luteolin 3-*O*-$\beta$-D-glucuronide | 18.06 | 3.44 | 9.20 | 239, 269, 345 | 461 $[M-1]^-$ |
| hesperidin | 19.80 | 4.01 | 21.17 | 284 | 609 $[M-1]^-$ |
| diosmin | 21.42 | 5.02 | 23.57 | 270, 345 | 609 $[M+1]^+$ |
| isoscutellarein 7-*O*-glucoside | 22.57 | 5.15 | 27.87 | 268, 337 | 465 $[M+1]^+$ |
| hispidulin 7-*O*-glucoside | 26.26 | 5.86 | 43.92 | 266, 334 | 463 $[M+1]^+$ |
| genkwanin | 42.62 | 19.50 | >150 | 269, 336 | 283 $[M-1]^-$ |

[a] *m*/*z* is the relationship between mass and charge.

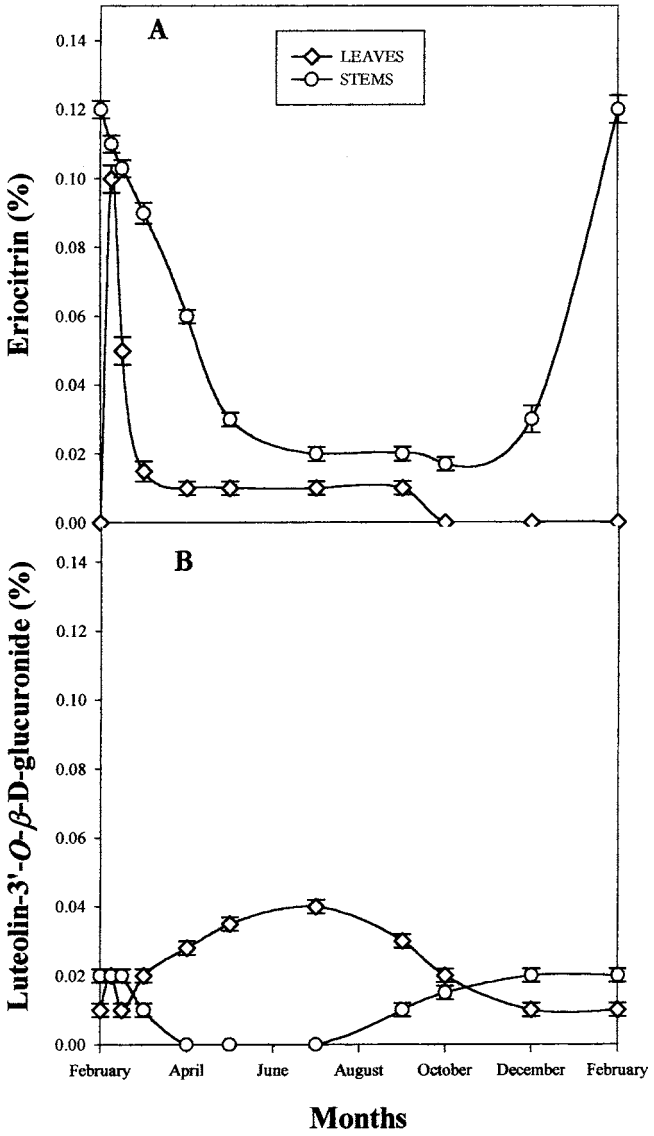


**Figure 4.** Changes in the mean total content (percent dry weight) of (**A**) eriocitrin and (**B**) luteolin 3′-*O*-$\beta$-D-glucuronide according to age in *R. officinalis* leaves and stems (2001–2002 season).

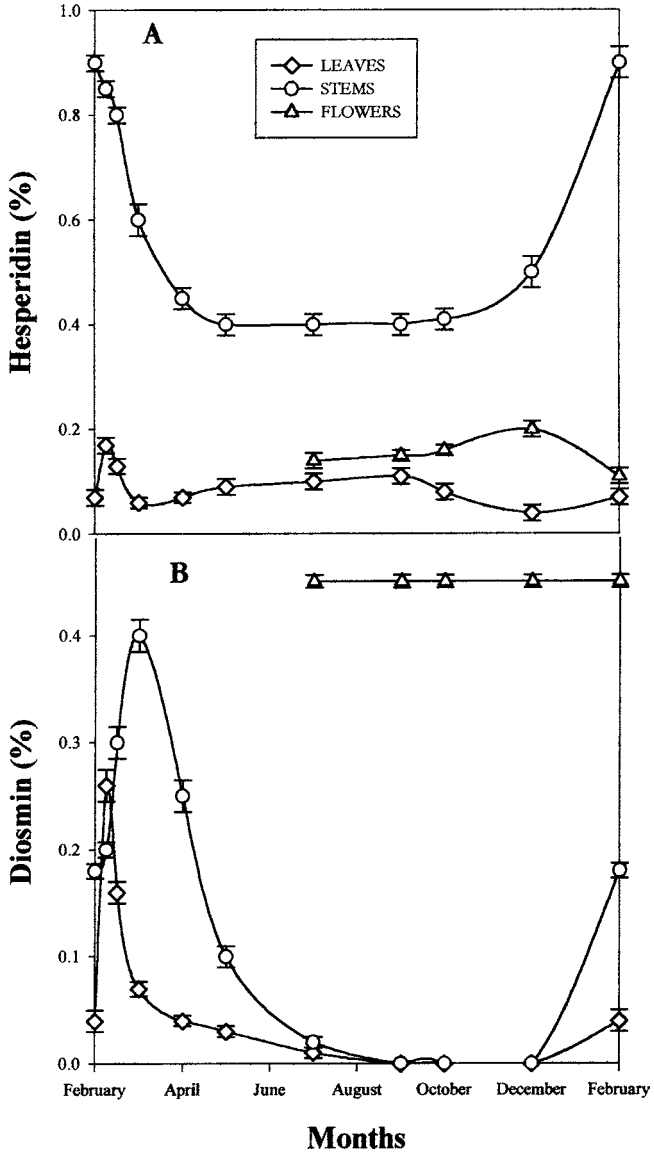


**Figure 5.** Changes in the mean total content (percent dry weight) of (**A**) hesperidin and (**B**) diosmin according to age in *R. officinalis* leaves, flowers, and stems (2001–2002 season).

min. The mobile phases were the same as for method 3 for flavonoids **1**−**6** and the same as for method 2 for genkwanin. The fractions were collected with a Pharmacia FRAC 100 (Pharmacia LKB Biotechnology, Uppsala, Sweden).

To confirm the structure of the main flavonoids located, the isolated compounds were identified by their $^1$H NMR (200 MHz) spectra (Bruker, Bremen, Germany) in DMSO-$d_6$ and mass spectra. Positive and negative ion electrospray ionization (ESI) mass spectra were recorded on a quadrupole Waters 4000 ZQ detector coupled to an HPLC system, model Alliance 2695 from Waters equipped with a UV−vis photodiode array detector model 2996. Experimental conditions for mass detection are shown in **Table 1**. The mobile phases were the same as for method 1, and the stationary phase was the same as for the chromatographic analysis.

## RESULTS AND DISCUSSION

**Identification of the Main Flavonoids in Leaves, Flowers, Stems, and Roots of *R. officinalis*.** HPLC analysis of DMSO extracts of leaves, flowers, stems, and roots of *R. officinalis* during growth pointed to the presence of many flavonoids. **Figure 2** shows the characteristic chromatogram of an extract from *R. officinalis* leaves, the organ that contains all of the

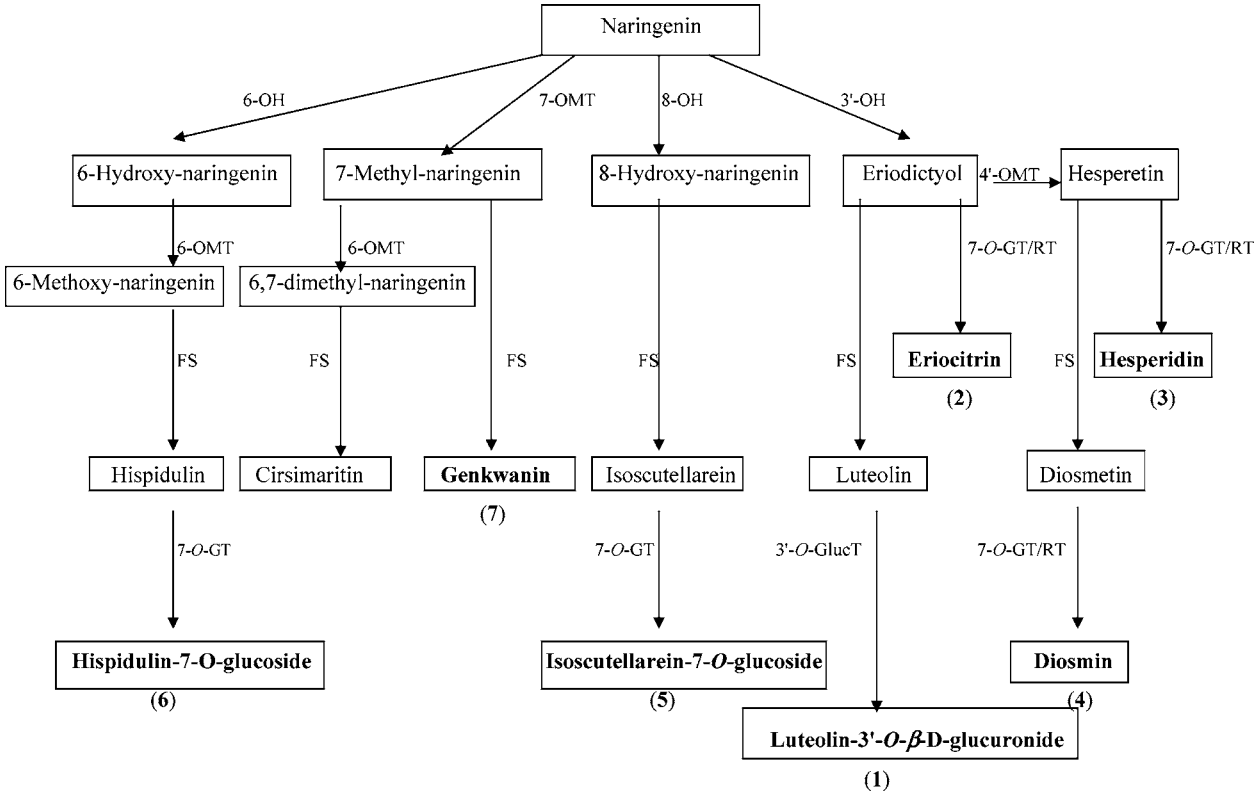


**Figure 6.** Possible interrelationship of flavonoids based on the distribution and proposed pathways for flavanone and flavone biosynthesis in *R. officinalis*. Enzymes involved are 6-*O*-methyltransferase (6-OMT), 7-*O*-methyltransferase (7-OMT), 4′-*O*-methyltransferase (4′-OMT), 6-hydroxylase (6-OH), 8-hydroxylase (8-OH), 3′-hydroxylase (3′-OH), 7-O-glucosyltransferase (7-*O*-GT), rhamnosyltransferase (RT), 3′-*O*-glucuronyltransferase (3′-*O*-GlucT), and flavone synthase (FS).

flavonoids studied, using method 1. The peaks for which retention times were identical to those of eriocitrin **(1)**, luteolin 3′-*O*-β-D-glucuronide **(2)**, hesperidin **(3)**, diosmin **(4)**, isoscutellarein 7-*O*-glucoside **(5)**, hispidulin 7-*O*-glucoside **(6)**, and genkwanin **(7)** are numbered. Peaks 5−7 are absent from the flower, stem, and root extracts, and peaks 1 and 2 are absent from the flower extract. **Table 2** shows the retention time ($t_R$) of each flavonoid in the three HPLC methods used in this study and the molecular ion peak (*m*/*z*).

Compounds **1**−**7** had mass and $^1$H NMR spectra identical with those of eriocitrin, luteolin 3′-*O*-β-D-glucuronide, hesperidin, diosmin, isoscutellarein 7-*O*-glucoside, hispidulin 7-*O*-glucoside, and genkwanin, respectively (*10*, *20*).

**Distribution of B-Ring 4′-Hydroxylated Flavonoids in *R. officinalis* Leaves, Flowers, Stems, and Roots during Plant Growth.** The presence of genkwanin and hispidulin 7-*O*-glucoside (also named homoplantaginin) was previously described in *R. officinalis* (*11*) and *Salvia officinalis* (*21*). In this study isoscutellarein 7-*O*-glucoside has also been identified. These three flavonoids reached maximum levels during the first stages of leaf growth (10−15 mm) (**Figure 3**). As occurs with the other rosemary polyphenolic compounds (*15*), the concentration decreased until leaf growth ended, probably due to the characteristic dilution process that occurs during this period, because the total amount per leaf remained constant (data not shown). Thereafter, the concentrations remained practically constant until the end of the cycle. In flowers, stems, and roots, no 4′-hydroxyflavones were detected. Their distribution suggests that only leaves are able to biosynthesize these flavonoids.

**Distribution of B-Ring 3′,4′-Dihydroxylated Flavonoids in *R. officinalis* Leaves, Flowers, Stems, and Roots during Plant Growth.** The presence of different luteolin glucuronides has been reported in *R. officinalis* (*10*, *11*) and other Labiatae plants (*8*, *13*). In this study, the flavone luteolin 3′-*O*-β-D-glucuronide and the flavanone eriocitrin were identified (**Table 2**). **Figure 4** shows the changes in the concentration of both flavonoids (percent dry weight) during the vegetative development of leaves and stems (2001−2002 season). These flavonoids were not present in flowers, whereas, in roots, the concentration ranged between 0.01 and 0.02%.

In leaves, the levels of eriocitrin increased during the first stages of growth, which suggests a biosynthesis with a maximum accumulation in the younger stage. The subsequent decrease in concentration was due to the above-mentioned dilution process during leaf growth. This behavior of flavonoids has been described by us previously in *R. officinalis* (*15*) and other plants (*22*−*24*). The content of eriocitrin in leaves remained constant until September, when it disappeared from these organs and simultaneously increased its presence in stems.

The maximum level reached by luteolin 3′-*O*-β-D-glucuronide in leaves during June−August suggests the existence of a delay between the activation of the enzymes involved in the flavanone and flavone biosynthesis. After eriodictyol (flavanone aglycone) biosynthesis, the transformation to eriocitrin by 7-*O*-glucosylation and rhamnosylation is practically immediate. However, luteolin (flavone) biosynthesis from eriodictyol shows a delay in its maximum accumulation rate and, consequently, the described glucuronide.

**Distribution of B-Ring 3′-Hydroxy- 4′-methoxylated Flavonoids in *R. officinalis* Leaves, Flowers, Stems, and Roots during Plant Growth.** Hesperidin was isolated from a rosemary leaf extract in previous studies (*10*) and its aglycon, hesperetin, was identified in *R. officinalis* (*11*) and *S. officinalis* (*21*). **Figure 5** shows the similar behavior of hesperidin and its corresponding

flavone, diosmin, to that of other compounds in *R. officinalis* during the first stages of leaf growth, with an early maximum followed by a decrease due to above-mentioned dilution process. The content of hesperidin in leaves then remained practically constant until September. It is interesting to note that the concentration of diosmin in flowers is higher than in other plant organs and remains constant as long as the flower is present on the plant.

The presence of hesperidin and diosmin in the vascular system is significant. Hesperidin shows even higher levels than the phenolic diterpenes and rosmarinic acid (*15*). In roots, the content of hesperidin varied between 0.25 and 0.35%, whereas diosmin showed levels of between 0.02 and 0.08%, neither being present at the end of the cycle.

**Flavonoid Translocation.** The possible existence of flavonoid transport through the vascular system of plants has been a controversial subject since the late 1960s. Macleod and Pridham (*25*) investigated the translocation rate of certain phenolic compounds introduced into the apical leaves of *Vicia faba*. It was found that kaempferol and quercetin were displaced at specific rates. In an attempt to detect naturally occurring phenols in the phloem fluid, aphids (*Macrosiphum pisi*) were placed on the stems of *V. faba*. Subsequent analysis of these fluids revealed the presence of tyrosine and two possible cinnamic acids but not flavonoids. However, several authors have suggested, and some of them have demonstrated, the existence of a transport mechanism for several polyphenolic compounds, including flavonoids (*26*−*29*). Our findings with *Citrus aurantium* plants have shown the presence of substantial concentrations of neohesperidin and naringin in the vascular fluids of the plant, suggesting that both flavonoids can be transported from the leaves to different plant organs (*22*).

The accumulation of flavonoids in leaves, flower buds, and fruits is the result of in situ biosynthesis and a translocation process from leaves to other leaves and organs. The regulation of this synthesis/transport relationship is probably an important factor in determining the final flavonoid content of the different plant organs, whereas the contribution of each process to the overall flavonoid content would vary during organ development (*23*, *29*). The presence of a substantial concentration of flavonoids, especially of the flavanones hesperidin and eriocitrin, and the flavone diosmin is evident in *R. officinalis* stems during the early stages of leaf development. The presence of these compounds in plant stems suggests an active transport process between the leaves of different development stages.

After the above-mentioned period, it is important to analyze the behavior of the most abundant flavonoids, hesperidin and diosmin, in stems, where the concentration of the former decreases at the end of leaf development, whereas the concentration of the latter falls later, particularly when the flowers start to develop, until it is practically absent from the vascular system. The concentration of diosmin is highest in flowers; diosmin distribution and the fact that its concentration remains constant during flower life suggest that the presence of this flavone in flowers is mainly the result of an important diosmin translocation from the leaves. In December, when the leaves sprout, the concentration of these flavonoids decreases slightly in leaves and, simultaneously, increases in stems. All such changes in flavonoid distribution could be related to the different processes of organ developments.

The relationship between flavonoids and vegetable hormones is well-known and, consequently, we might consider the existence of a relationship between the levels of flavonoids and phytoregulators during the plant's development. There are some studies (*30*−*33*) on the relationships between flavonoids, hormones (especially auxins), and the enzymes responsible for the oxidation of these hormones. These studies reported that the 4′-hydroxylated flavonoids activate the oxidation of auxins, whereas 3′,4′-dihydroxylated flavonoids and, especially, 3′-hydroxy-4′-methoxylated flavonoids act as inhibitors of the enzymes responsible for that oxidation, particularly the glycosylated forms. The distribution of flavonoids observed in *R. officinalis* agrees with this hypothesis (hesperidin and diosmin are 3′-hydroxy-4′-methoxy flavonoids), suggesting in this plant a functional and structural relationship between phytoregulators and flavonoids, where flavonoids would be "protectors" of the activity of phytoregulators.

**Postulation of a Biosynthetic Pathway for Flavonoids in *R. officinalis*.** The presence and distribution of flavonoids in the different organs of the plant that have been studied in this investigation, together with the papers mentioned, lead us to propose a hypothesis for the general pathway of biosynthesis of these compounds in the Labiatae plants.

Possible biosynthetic pathways of flavonoids in *R. officinalis* are shown in **Figure 6**. Some of the flavonoids involved in this pathway have been isolated and identified in rosemary and other Labiatae, and others have not been reported; however, their structures might make them intermediates in the described pathway. Three different steps can essentially be distinguished in this complex scheme: the A- and B-ring modifications (hydroxylation and methylation) that contribute to the formation of several flavanones; the conversion of these flavanones to the corresponding flavones (via flavone synthase); and, finally, the subsequent glycosylation of these flavones and flavanones, including glucuronidation reactions. This theoretical biosynthesis scheme is supported by the data of rosemary flavonoid distribution (*8*−*15*, *21*) and by the previous findings on flavonoid biosynthesis in several plant species (*24*, *34*, *35*). Final proof of this hypothesis warrants more research to isolate the different enzymes involved in this biosynthesis scheme.

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