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(+)-Methyl Jasmonate-Induced Bioformation of Myricetin, Quercetin and Kaempferol in Red Raspberries

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The effect of postharvest treatment with enantiomers of methyl jasmonate (MJ) in conjunction with ethanol on bioformation of myricetin, quercetin and kaempferol in red raspberry was studied. For comparison, postharvest treatment with the commercial stereoisomeric mixture of MJ in conjunction with ethanol was simultaneously accomplished. The levels obtained were contrasted with those determined in untreated (control) samples. Exogenous (+)-MJ induced an enhancement in the levels of myricetin, quercetin and, particularly, kaempferol whereas the exposition to (−)-MJ exhibited the opposite effect. Enzymatic assays were carried out in presence and absence of (−)-MJ and (+)-MJ to evaluate possible changes in the activity of the enzymes regulating the bioformation of flavonols in red raspberries as a consequence of the treatments. From the results of the assays both (−)-MJ and (+)-MJ inhibited the activity of flavanone 3β-hydroxylase (FHT) and flavonol synthase (FLS), which are directly involved in the formation of flavonols from (−/+)-naringenin. From these results, it is speculated that the activity of phenylalanine ammonia lyase (PAL) regulating the formation of (−/+)-naringenin from L-phenylalanine by (+)-MJ in conjunction with ethanol is promoted. Postharvest treatment of red raspberry with (+)-MJ in ethanol is proposed as a mean to increase flavonol content in red raspberries.

KEYWORDS: Methyl jasmonate; enantiomers; flavonols; red raspberry; enzyme assay

INTRODUCTION

Flavonols (e.g., myricetin, quercetin and kaempferol) are plant phytochemicals that attract scientists' interest for their potential chemopreventive effects consisting in their ability to arrest and/or reverse the progression of malignant cells (*1*, *2*). In vitro model studies for heart disease suggest that myricetin, quercetin and kaempferol can be more powerful antioxidants than traditional vitamins (*3*). Similarly, recent epidemiological and experimental studies indicate that flavonols can also act as important proteasome inhibitors and apoptosis inductors (*4*). Quercetin, in particular, exhibits anti-inflammatory (*5*), antitumor (*6*) and antihypertensive effects (*7*). As a consequence, long-term high consumption of flavonol-rich foods is related to a lower risk of developing certain diseases such as coronary heart disease (*8*), stroke (*9*), lung cancer (*10*), and stomach cancer (*11*), among others.

In this context, edible berries have been reported to be one of the most important sources of flavonols (*12*). The formation of flavonols myricetin, quercetin and kaempferol in berries (i.e., strawberries) has been reported to occur through the phenylpropanoid metabolism (*13*). Specifically, naringenin is transformed in quercetin and kaempferol by means of the enzyme flavanone 3β-hydroxylase (FHT), which leads to dihydroflavonols (*13*−*15*). A further enzyme, flavonol synthase (FLS), may subsequently oxidize dihydroflavonols to flavonols. At the same time, phenylalanine ammonia lyase (PAL) catalyzes the formation of naringenin from phenylalanine. A similar formation of kaempferol from naringenin has also been described in *Citrus unshiu* (*16*). However, in this latter study the asymmetric character of naringenin is particularly relevant in such a way that kaempferol is synthesized from both (*R*)- and (*S*)-dihydroflavonols, which are in turn formed from (*R*)- and (*S*)-naringenin by the action of FLS and FLS + FHT, respectively. Regarding myricetin, although it is also produced through the phenylpropanoid metabolism, its synthesis occurs in general terms further in the pathway (*17*). However, the bioformation of myricetin, quercetin and kampferol in raspberries has not been studied as yet.

Nowadays the interest in developing health-promoting foods by the enhancement of the content of their bioactive components is undoubted. In this respect, some natural volatile compounds, such as methyl jasmonate (MJ) and ethanol, have been reported to increase the content of a number of bioactive plant components. Specifically, there are studies concerning the impact of exogenous MJ, alone or in conjunction with ethanol, on flavonoid content, antioxidant capacity, quality and postharvest life of various berries (*18*−*20*). In addition, the modification of the activity of enzymes involved in the metabolism of flavonoids by the exogenous application of (−/+)-MJ has been occasionally reported in the literature. Particularly, Kim et al. (*21*) have proven the impact of (−/+)-MJ on the promotion of the activity of PAL,

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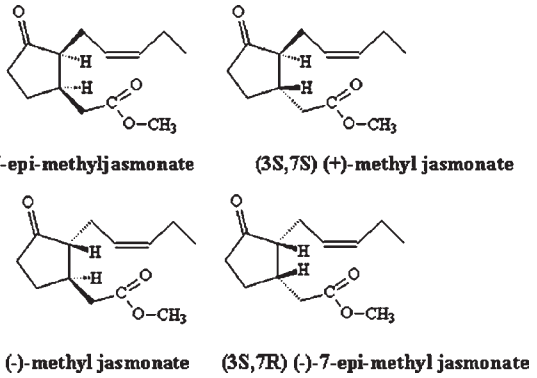

Figure 1. Chemical structure of four steroisomers of methyl jasmonate.

which is regarded as a key regulatory enzyme in the production of phenolic compounds in radish sprout. The exposition to MJ and ethanol promotes the accumulation of phenolic compounds by triggering the phenylpropanoid metabolism through an increase in PAL activity in carrots (*22*). Other researchers have demonstrated that (−/+)-MJ treatment can effectively enhance chilling tolerance and reduce chilling injury of loquat fruit by means of the enhancement of the antioxidant enzyme activity (*23*).

Whereas ethanol is an achiral molecule, MJ has two chiral centers at C-3 and C-7 in such a way that it can exist as four different stereoisomers (**Figure 1**). Interestingly, different mechanisms of action for various biological activities have been described for the four MJ stereoisomers (*24*, *25*). Despite the importance of the stereochemistry of the molecule of MJ for its biological activity, all the studies carried out with MJ have been always focused on the effect of the commercial stereoisomeric mixture due probably to the unavailability of the pure MJ enantiomers. The impact of the treatment with MJ individual stereoisomers on food components has not been investigated to date.

We here intended to increase the levels of flavonols myricetin, quercetin and kaempferol during the postharvest life of red raspberry fruits by carrying out three different treatments: the exogenous application of (−/+)-MJ, (−)-MJ and (+)-MJ vapors, respectively, in conjunction with ethanol. Our intention was to overcome the limitations earlier found by treating strawberries only with (−/+)-MJ (*20*). A further purpose was to get a more comprehensive insight into the effect of the enantiomers of MJ on the bioformation of flavonols myricetin, quercetin and kaempferol in raspberry fruits. To that end, assays for the activity of FHT and FLS enzymes, which are directly involved in flavonol synthesis, were also accomplished.

MATERIALS AND METHODS

Chemicals and Samples. HPLC grade methanol (MeOH) and acetonitrile (ACN) were supplied by Labscan Ltd. (Dublin, Ireland). Trifluoroacetic acid (TFA), MJ, (−/+)-naringenin, polyclar AT, Tris/HCl, $FeSO_4$, Na-ascorbate, 2-oxoglutarate, ethylenediaminetetraacetic acid (EDTA) and the flavonol standards myricetin, quercetin, kaempferol and morin were purchased from Sigma (Steinheim, Germany). MJ was obtained as a stereoisomeric mixture made up of 45% of each (+)- and (−)-MJ and 5% of each (+)- and (−)-epiMJ. *tert*-Butylhydroquinone (TBHQ) and ethyl acetate were provided by Fluka (Steinheim, Germany) and Scharlau, respectively. Milli-Q water was collected from a purification system (Millipore, Milford, MA). Full-ripe red raspberry fruits (variety Glen Lyon, Huelva, Spain) were acquired from the local supermarket. Fresh berries with uniform size, color, and ripeness and free from damage were used for the treatments and enzyme preparations. Once acquired, they were immediately treated with (−/+)-MJ, (−)-MJ and (+)-MJ vapors. (−)-MJ and (+)-MJ were isolated from the commercial (−/+)-MJ as explained below.

Isolation of MJ Enantiomers by HPLC-SPE. Pure (−)- and (+)-MJ were obtained from standard MJ by following the HPLC-SPE method developed elsewhere (*26*). In brief, the HPLC equipment used was a Hewlett-Packard model 1050 (Wilmington, DE) chromatograph fitted with a manual injection valve (model 7125, Rheodyne, Cotati, CA) having a 20 μL sample loop and an ultraviolet (UV) detector operated at 210 nm. To perform the resolution of MJ enantiomers a 200 × 4.0 mm i.d. column packed with a 5 μm layer of permethylated β-cyclodextrin (Nucleodex β-PM, Macherey-Nagel, Düren, Germany) was utilized. HPLC fractions for both (−)- and (+)-MJ were simultaneously accumulated to gather a sufficient amount of each enantiomer to carry out the treatments. The fractions collected were then concentrated by SPE as earlier reported (*26*) with the exception of the employment of ethanol instead of chloroform as the elution solvent. The final concentration of the fractions containing (−)- and (+)-MJ was 8.9×10^{-4} M in EtOH for each enantiomer, respectively. Raspberry fruits were then treated with these two fractions. For comparison, treatment with 8.7×10^{-4} M EtOH of commercial (−/+)-MJ was also carried out. Additionally, HPLC fractions containing 8.9×10^{-4} M of (−)- and (+)-MJ in EtOH were subsequently collected to accomplish the enzyme assays, as specified below.

Treatments. Three different treatments were carried out by using (−/+)-, (−)-, and (+)-MJ. A 120 g weight of raspberries (approximately 40 berries) was distributed in three different 600 mL containers (40 g of berries in each) to accomplish the three different treatments. A 0.040 μL volume of MJ standard in 1 mL of ethanol and the (−)- and (+)-MJ fractions isolated by HPLC-SPE (i.e., 0.020 μL of each enantiomer in 1 mL of ethanol) were respectively placed into three different vials, which were, in turn, placed inside of each of the three containers whose lids were screwed. (−/+)-, (−)- and (+)-MJ were allowed to spontaneously vaporize during 24 h at 25 °C. After treatments, the vials containing (−/+)-, (−)- and (+)-MJ were immediately withdrawn before storage of the samples. The three containers were subsequently kept at 10 °C for 7 days. A 40 g weight of raspberries (approximately 20 berries) was also placed in another container to be used as a control. The procedure applied to the control samples was exactly the same as that applied to the MJ-treated samples with the only exception being the use of an empty vial instead of a MJ vial. As explained below, flavonol content was measured on days 5 and 7 after treatments. In addition, it was also determined on the treatment day (so-called day 0) to know the composition of the raspberry samples in the starting point. The analyses of the control and treated samples were accomplished in duplicate each day.

Determination of Flavonol Content. *Extraction and Hydrolysis*. The isolation of myricetin, quercetin and kaempferol from raspberries, untreated and treated with (−/+)-, (−)- and (+)-MJ, was carried out as follows: a 10 g weight was homogenized with a blender. Acidified methanol (25 mL) containing 1% (v/v) HCl, 3.0×10^{-3} M TBHQ and the internal standard (morin, 0.5 μg) were added to the sample. Subsequently, HCl (1.2 M, 5 mL) was added to the mixture, which was then stirred at 90 °C under reflux for 2 h to hydrolyze flavonol glycosides to the corresponding aglycons. The resulting extract was allowed to get cold and then centrifuged at 1500*g* (15000 rpm) for 10 min. The upper layer was taken, filtered through a 0.45 μm filter (Millipore) and analyzed by HPLC as explained below.

HPLC Analysis. The same liquid chromatograph as that used to isolate MJ enantiomers was employed. The separation of myricetin, quercetin, kaempferol and the internal standard (morin) was accomplished on an ODS reverse phase (C18) column (250 mm ×4.6 mm i.d., 5 μm particle size, ACE, Madrid, Spain). The elution was performed by using solvent A (H_2O containing 0.1% TFA) and solvent B (ACN/MeOH, 80/20, v/v) at a constant flow rate of 1.2 mL/min. A linear gradient was applied from the initial eluent composition (A:B, 70:30) up to A:B, 60:40 (v/v) for the first 5 min and then within 2 min up to final composition (A:B, 50:50, v/v), which was maintained for 13 min. The ultraviolet (UV) detector operated in all instances at 360 nm. Data acquisition was carried out by using Agilent ChemStation (Rev. A 10.02, 1757). Identification of myricetin, quercetin and kaempferol was based on the comparison between the retention times obtained from the standards and from the chromatographic signals in the samples run under the same experimental conditions. Spiked extracts were additionally analyzed to confirm the identity of the target flavonols. Relative areas of the target flavonols with respect to that of morin were used to estimate their contents. The extraction and

Table 1. Content[a] (Expressed as μg/g Fresh Weight ± Standard Deviation) of Myricetin, Quercetin and Kaempferol in Red Raspberry Samples Untreated (Control) and Treated with (−/+)-MJ on Days 0, 5, and 7 after Treatment[b]

		control		(−/+)-MJ treated	
flavonols	day 0	day 5	day 7	day 5	day 7
myricetin	0.877 ± 0.061	0.738 ± 0.073	0.468 ± 0.030	0.654 ± 0.068	0.546 ± 0.070
quercetin	0.551 ± 0.053	0.499 ± 0.060	0.220 ± 0.042	0.487 ± 0.051	0.257 ± 0.033
kaempferol	0.180 ± 0.013	0.080 ± 0.007	0.057 ± 0.004	0.043 ± 0.006	0.042 ± 0.002

[a] Mean value (n = 2) ± SD. [b] The values shown have been obtained from two replicates.

Table 2. Content[a] (Expressed as mg/g Fresh Weight ± Standard Deviation) of Myricetin, Quercetin and Kaempferol in Raspberry Samples Untreated (Control) and Treated with (−)-MJ on Days 0, 5, and 7 after Treatment[b]

		control		(−)-MJ treated	
flavonols	day 0	day 5	day 7	day 5	day 7
myricetin	0.118 ± 0.001	0.109 ± 0.032	0.090 ± 0.042	0.092 ± 0.006	0.046 ± 0.004
quercetin	0.031 ± 0.002	0.020 ± 0.002	0.012 ± 0.001	0.022 ± 0.001	0.005 ± 0.002
kaempferol	nd[c]	0.007 ± 0.001	0.010 ± 0.001	nd	0.003 ± 0.003

[a] Mean value (n = 2) ± SD. [b] The values shown have been obtained from two replicates. [c] Not detected.

Table 3. Content[a] (Expressed as mg/g Fresh Weight ± Standard Deviation) of Myricetin, Quercetin and Kaempferol in Raspberry Samples Untreated (Control) and Treated with (+)-MJ on Days 0, 5, and 7 after Treatment

		control		(+)-MJ treated	
flavonols	day 0	day 5	day 7	day 5	day 7
myricetin	0.432 ± 0.051	0.197 ± 0.033	0.016 ± 0.010	0.289 ± 0.021	0.098 ± 0.016
quercetin	0.591 ± 0.042	0.173 ± 0.014	0.031 ± 0.003	0.325 ± 0.001	0.110 ± 0.021
kaempferol	0.172 ± 0.038	0.139 ± 0.005	0.015 ± 0.001	0.220 ± 0.002	0.132 ± 0.041

[a] Mean value (n = 2) ± SD.

subsequent HPLC analysis of myricetin, quercetin and kaempferol were carried out in duplicate for each sample. The analytical approach described for the determination of the studied flavonols was equally applied to control and treated raspberries.

Measurement of FHT and FLS Activities. *Enzyme Preparation.* The raspberries were first frozen at −80 °C and lyophilized. Subsequently, the crude enzymes were extracted as follows: the fruits were ground in a mill to a fine powder. A total of 1.5 g of the powder obtained, 0.25 g of quartz sand, 0.25 g of Polyclar AT and 4 mL of 0.1 M Tris/HCl (containing 0.4% Na-ascorbate, pH 7.25) were properly homogenized. The resulting homogenate was then centrifuged at 4 °C and 5000*g* (15000 rpm) for 10 min. Finally the supernatant was taken to accomplish both FHT and FLS assays, as detailed below.

Enzyme Assays. The FHT and FLS activities were simultaneously measured by mixing 9.1 nmol of (−/+)-naringenin, 50 μL of the enzyme preparation obtained above, 5 μL of 11.23 mM 2-oxoglutarate, 5 μL of 6.47 mM $FeSO_4 \cdot 7\ H_2O$, and 12.5 μL of a buffer made up of 0.1 M Tris/HCl + 0.4% Na-ascorbate (pH 7.25). When the enzyme assay was performed in presence of (−)- or (+)-MJ, 1 μL of the collected fraction corresponding to each enantiomer was added. The enzyme assay was then incubated for 15 min at 30 °C. After that, the assay was terminated by addition of 140 μL of ethyl acetate, 10 μL of acetic acid and 10 μL of 0.1 mM EDTA. The organic phase was analyzed by HPLC analysis as detailed below.

HPLC Analysis. Chromatographic analyses of the content of flavonols resulting from the enzyme assays in absence and presence of (−/+)-, (−)- and (+)-MJ were performed using a Konik model 560 (Konik, Sant Cugat del Vallés, Barcelona, Spain) chromatograph fitted with a manual injection valve (model 7725i, Konik, Sant Cugat, Barcelona, Spain) having a 20 μL sample loop and a column thermostat, which was kept at 25 °C at all times. The chromatographic signals corresponding to (−/+)-naringenin and flavonols myricetin, quercetin, kaempferol and the internal standard (morin) were detected by using an ultraviolet (UV) detector, whose wavelength was set at 290 nm for (−/+)-naringenin detection and 360 nm for flavonols. The separation of (−/+)-naringenin and flavonols was carried out on a ODS reverse phase (C18) column (150 mm ×4.0 mm i.d., 5 μm particle size, Teknokroma, Sant Cugat, Barcelona, Spain). The elution was performed by using solvent mixture made up of solvent A (ACN) and solvent B (H_2O containing 0.1% TFA) at a constant flow rate of 1.2 mL/min. A linear gradient was applied from the initial eluent composition (A:B, 30:70), which was maintained for 5 min, and then it increased up to a composition A:B, 42:58 (v/v) for the following 9 min. This percentage was maintained for another 5 min. Data acquisition was carried out by using Konikrom Plus (KNK-725−240). Identification of (−/+)-naringenin, myricetin, quercetin kaempferol and morin was based on the comparison between the retention times obtained from the standards and from the chromatographic signals in the samples run under the same experimental conditions. Each assay was injected three times. For comparison, the enzyme activity was also measured in absence of MJ to be used as a control. The LC equipment was washed by passing acetonitrile through the sample path for 15 min after every run.

Statistical Analyses. Analysis of variance (ANOVA) of data on the influence of (−/+)-, (−)- and (+)-MJ in ethanol on flavonols was performed using StatGraphics 5.1. The effect of the treatments on the levels of myricetin, quercetin and kaempferol was assessed by the Fisher test. Differences between data were compared by least significant differences (LSD). The values used were always the mean of the two replicates performed. Differences at $p \leq 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

As detailed in Blanch et al. (*26*), by applying the HPLC-SPE method developed, 0.89×10^{-2} M of (−)- and (+)-MJ in EtOH with enantiomeric purity close to 100% were separately obtained. These two fractions together with a similar concentration of the stereoisomeric mixture of MJ in ethanol were used to perform the three treatments.

Tables 1, **2** and **3** indicate the content (expressed as μg/g fresh weight) of myricetin, quercetin and kaempferol in red raspberries untreated (i.e., control) and treated with (−/+)-, (−), and (+)-MJ in conjunction with ethanol vapors, respectively, on days 5 and 7 after each treatment. In addition to measurements on days 5 and 7

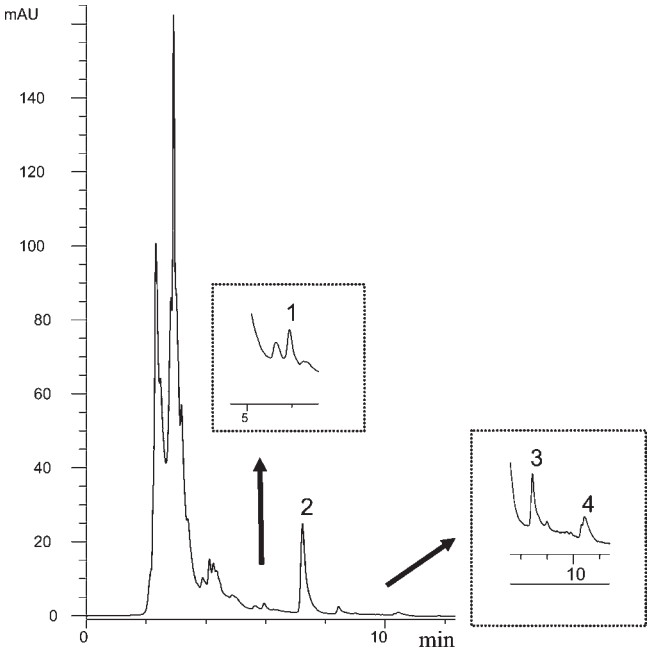

Figure 2. Chromatogram obtained from the HPLC analysis of myricetin, quercetin and kaempferol in untreated raspberry fruits on day 0. Peak identification: (1) myricetin, (2) internal standard (morin), (3) quercetin, (4) kaempferol.

after treatment, flavonol content was also measured on day 0 as a reference. As an example, **Figure 2** illustrates the chromatogram obtained from the analysis of control raspberries on day 0. The relative standard deviation (RSD) of the overall approach described in Materials and Methods, including the extraction followed by the HPLC analysis of flavonols in untreated raspberries, was 10%, 18% and 15% for myricetin, quercetin and kaempferol, respectively. Taking into account that different berries have to necessarily be used to extract the target compounds in each analysis, the RSD values obtained were attributed to the natural variability and, therefore, the repeatability of the analytical method used was considered satisfactory. To estimate flavonol content, linear regression was applied. Standard solutions of concentrations ranging from 1.6 to 160 M of the three studied flavonols were prepared. The regression equation obtained was $y = 0.0501x + 0.0909$ with a correlation coefficient (r^2) of 0.998, 0.994, and 0.996 for myricetin, quercetin and kaempferol, respectively.

As observed in **Table 1**, the treatment with the stereoisomeric mixture of (−/+)-MJ in conjunction with ethanol did not result in significant changes ($p > 0.05$) in the content of myricetin, quercetin and kaempferol on either day 5 or day 7 after the treatment. Whereas the content of kaempferol decreased slightly as a consequence of (−/+)-MJ treatment, the levels of myricetin and quercetin in the treated raspberries barely varied with respect to those in the control samples on both days. On the other hand, the amounts of the three flavonols measured on day 0 reflected a continuous and regular decline during the 7 day storage, which was equally observed in control and treated samples. The impact of (−/+)-MJ on flavonols has been occasionally reported in the literature. In this respect, the scarce studies dealing with MJ effect on flavonols have been particularly focused on quercetin. Particularly, MJ treatment has been reported to reduce the content of quercetin in tomato leaves, its amount reaching only 11−22% of the control value after the 2-week treatment (*27*). Similarly, other researchers have also described that the production of quercetin is not enhanced either in "Fuji apples" by MJ treatment (*28*). In this line, a previous study on the influence of the stereoisomeric mixture of (−/+)-MJ on myricetin, quercetin and kaempferol contents in strawberry fruits carried out in our laboratory suggested a constancy in the amounts of these flavonols whatever experimental conditions were applied (*20*).

In general, the levels of flavonols detected in untreated raspberries in the present study were always <5.0 μg/g. Flavonol contents in berries have been reported in the literature in the same interval as the amounts here reported (*12*). In this context, it is important to point out that flavonol contents vary within a considerably wide range in nature. Increasing data suggest that several aspects including the genotype, geographical area and cultivar influence the levels of flavonols (*29*, *30*). The trend of flavonols to decline during the postharvest storage of control raspberries supports data in the literature on the slight "increase of flavonol content between unripe and pre-ripe stages followed by a gradual diminishment as ripening progresses (i.e. from ripe to soft fruit)" (*31*).

In line with the results of (−/+)-MJ treatment, the levels of myricetin, quercetin and kaempferol did not increase either after the postharvest application of the (−)-enantiomer of MJ with ethanol to red raspberries (see **Table 2**). Actually, a comparison between control and (−)-MJ-treated samples showed relative steadiness in the content of the three flavonols on day 5. Specifically, myricetin, quercetin and kaempferol kept their content constant around 0.100, 0.020, and 0.007 mg/g, respectively. This steadiness became a decrease on day 7 after (−)-MJ treatment since myricetin content diminished from 0.090 to 0.046 mg/g as a consequence of the application of (−)-MJ. The same thing happened to quercetin and kaempferol, which decreased from 0.012 to 0.005 mg/g and from 0.010 to 0.003 mg/g by comparing control and (−)-MJ treated samples. The diminishment in flavonol content as a result of (−)-MJ treatment on day 7 was statistically significant ($p < 0.05$). Regarding the evolution of flavonols during storage, in accordance with the trend earlier observed, the levels decreased from day 0 to day 7 in both control and (−)-MJ-treated samples. Specifically, the content of myricetin diminished 8% and 24% on days 5 and 7, respectively, with respect to day 0 in control samples and 23% and 62% in treated samples. A similar effect was observed for quercetin, which decreased 36% and 32% on days 5 and 7 respectively in control samples and 30% and 85% in treated raspberries. Kaempferol content did not actually decrease since it was barely detected in all instances.

As seen in **Table 3**, contrary to the two previous treatments, the postharvest exposition of red raspberry fruits to (+)-MJ vapor led to a general enhancement of the levels of myricetin, quercetin and kaempferol. The content of the three flavonols underwent a significant increase ($p < 0.05$) after the (+)-MJ treatment on both days 5 and 7 with respect to control samples analyzed on the same days. In particular, (+)-MJ treatment resulted in an increase in the content of myricetin of 32% and 85% on days 5 and 7, respectively. Enhancements of 47% and 72% for quercetin and of 37% and 89% for kaempferol were equally attained on days 5 and 7 respectively after the application of (+)-MJ vapor to red raspberries. Conversely, the natural evolution of the content of myricetin, quercetin and kaempferol confirmed the diminishment from day 0 to day 7 observed in the other two treatments. As mentioned in the Introduction, no studies about the effect of MJ enantiomers on foods can be found in the literature.

Table 4 summarizes the effect of the three treatments, (−/+)-MJ, (−)-MJ and (+)-MJ, on the contents of myricetin, quercetin and kaempferol in red raspberries on days 5 and 7 after the treatments considering statistical significance.

Table 4. Summary of the Effects of (−/+)-MJ, (−)-MJ and (+)-MJ Treatments on the Contents of Myricetin, Quercetin and Kaempferol in Red Raspberries on Days 5 and 7 after the Treatments. The Same Effects Were Observed for the Three Flavonols Studied.

(−/+)-MJ	(−)-MJ	(+)-MJ
• No significant ($p > 0.05$) effect either on day 5 or day 7	• No significant effect ($p > 0.05$) on day 5 • Significant decrease on day 7 ($p < 0.05$)	• Significant increase ($p < 0.05$) on both days 5 and 7

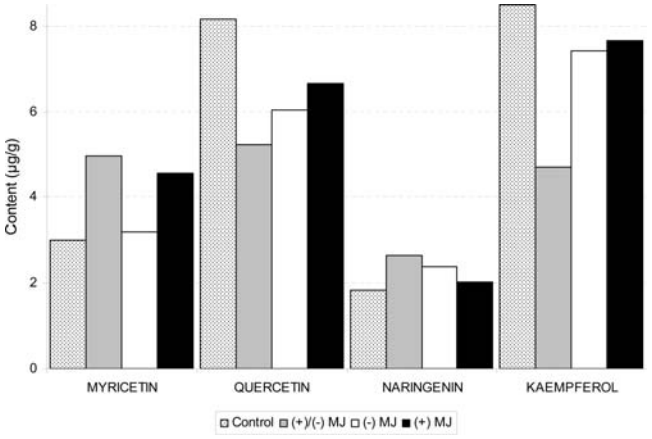

Figure 3. Content (expressed as μg/g fresh weight) of myricetin, quercetin, naringenin and kaempferol in the enzyme assays in the presence of (−/+)-MJ, (−)-MJ and (+)-MJ, respectively.

Considering the different biological properties attributed to the stereoisomers of MJ in the literature (*24*, *25*) as well as the influence of (−/+)-MJ on enzyme activities (*21*−*23*), it is speculated that (+)-MJ might be able to promote the activity of some enzymes directly involved in flavonol biosynthesis and, therefore, enhance flavonol content. On the contrary, (−)-MJ might act as an inhibitor resulting in the significant decline observed on day 7. This would explain why the application of exogenous (−/+)-MJ, which is a mixture of both (−)- and (+)-MJ, did not affect the levels of myricetin and quercetin (just a slight decrease in kaempferol content) either on day 5 or on day 7.

Enzymatic studies were scheduled to evaluate the influence of MJ enantiomers on the enzymes regulating the formation of flavonols from (−/+)-naringenin in raspberries. The enzymes FHT and FLS were simultaneously investigated for their activities in presence of (−/+)-, (−)- and (+)-MJ. As also mentioned in Materials and Methods, the enzymatic reaction was equally accomplished in the absence of MJ to be used as a control.

Figure 3 illustrates the contents (μg/g fresh weight) of myricetin, quercetin, (−/+)-naringenin and kaempferol obtained from HPLC analysis of the enzyme assay in the absence of MJ (control) and in the presence of (−/+)-, (−)- and (+)-MJ, respectively. In contrast to the results obtained from the determination of flavonol content, all the treated samples resulted in lower content of quercetin and kaempferol than the control samples in all cases, varying between 25% and 35% decrease for quercetin and between 15% and 45% for kaempferol according to the specific treatment applied. These lower contents cannot be attributed to the natural variability, which, as previously mentioned, was 18% and 15% for quercetin and kaempferol, respectively. Therefore, the diminishment in quercetin and kampferol contents might indicate that both (−)- and (+)-MJ (and therefore their stereoisomeric mixture, (−/+)-MJ) inhibit the activity of FHT and FLS enzymes controlling the formation of quercetin and kaempferol from (−/+)- naringenin in raspberries. This fact was confirmed by the content of (−/+)-naringenin found, which was always lower in the control sample.

Since both (−)- and (+)-MJ exhibited an inhibitory effect on FHT and FLS activities in raspberries, it is deduced that the higher content of flavonols measured in (+)-MJ treated berries is probably due to a greater promoting effect of (+)-MJ on PAL activity, which regulates the formation of (−/+)-naringenin from L-phenylalanine, than inhibitory effect on FHT and FLS activities. This would lead to a larger amount of (−/+)-naringenin in berries treated with (+)-MJ and, consequently, to larger contents of quercertin and kaempferol. Actually, this is not surprising considering, on the one hand, that the PAL activity has been reported to be increased in radish by 60% at 24 h after (−/+)-MJ treatment (*21*) and in carrots when (−/+)-MJ is synergistically used with wounding (*22*) and, on the other hand, the asymmetric character of PAL enzyme. In this respect, it is important to bear in mind that the transformation of L-phenylalanine into (−)- and (+)-naringenin occurs through a stereoselective mechanism (*13*, *27*).

As far as the distinct treatments are concerned, a comparison between them shows that (+)-MJ resulted approximately in 10% higher contents of quercetin and kaempferol than (−)-MJ (see **Figure 3**), which is indicative of a greater inhibitory effect of (−)-MJ than (+)-MJ on FHT and FLS activities. This observation was confirmed with the higher amount of (−/+)-naringenin when the enzyme assay was carried out in the presence of (−)-MJ than when (+)-MJ was used. Besides, as also observed in **Figure 3**, the contents of the target compounds from the enzyme assay with (−/+)-MJ correspond to the sum of effects obtained from (−)- and (+)-MJ considered individually.

Regarding the results of the assay for myricetin, a different tendency was clearly observed. The addition of (+)-MJ to the enzyme assay appeared to activate the formation of myricetin whereas the presence of (−)-MJ did not have a significant effect. As earlier explained, myricetin is not directly formed from (−/+)-naringenin, being further in the phenylpropanoid pathway (*17*). Therefore, it is likely that the variation in its content is largely dependent on the amounts of quercetin and kaempferol formed during the assay rather than the FHT and FLS activities.

In conclusion, the postharvest treatment of raspberry fruits with (+)-MJ in conjunction with ethanol enables the content of myricetin, quercetin and kaempferol in raspberry fruits to be significantly ($p < 0.05$) increased. Assays for the FHT and FLS activities in the presence and absence of (−)- and (+)-MJ suggests an inhibitory effect of both enantiomers on these enzymes in red raspberry. The promoting effect of (+)-MJ on PAL regulating the formation of (−/+)-naringenin from L-phenylalanine activity is speculated. Specific assays for PAL activity would be necessary to confirm this speculation. This is part of the aim of a further study. The enhancement in biological properties of raspberries caused by (+)-MJ in conjunction with ethanol treatment could provide more antioxidant to regular diets, at low cost, and be an alternative to genetic modifications and breeding activities. Biological studies in this respect are currently in progress to evaluate this aspect.

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