DOI:10.1021/jf9011455

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Sorption and Interaction of the Flavonoid Naringenin on Tomato Fruit Cuticles

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The flavonoid naringenin accumulates in tomato fruit epidermis during ripening. The sorption of this flavonoid to enzymatically isolated cuticles of *Solanum lycopersicum* was studied as a function of the temperature and naringenin concentration at two stages of fruit growth. The selected stages were mature green, without flavonoids in the cuticle, and ripe tomato, with significant amounts of flavonoids in the cuticle. Sorption isotherms showed different behaviors that could be explained in terms of different affinities of the sorbed flavonoid for the cuticular matrix. The partition coefficient of naringenin in the system cuticle/water solution was a function of temperature and concentration. Changes in the free energy, enthalpy, and entropy for the phase transfer of naringenin to cuticle were also calculated, indicating the existence of naringenin−naringenin interactions replacing naringenin−cuticular matrix interactions at high concentrations with the final result of solid precipitations in the form of clusters within the cutin matrix.

KEYWORDS: Tomato fruit; cuticle; flavonoids; naringenin; sorption

INTRODUCTION

The leaf and fruit surfaces of higher plants are covered by the cuticle or cuticular membrane. Thus, the plant cuticle constitutes the interface between the plant tissue and the environment and, quantitatively, this cuticular material occurs in large amounts in both natural and agricultural plant communities: between 180 and 1500 kg/ha (*1*). From a chemical point of view, the cuticle is formed by an insoluble polymer matrix of polyhydroxy-fatty acid esters called cutin. Associated with this biopolymer are waxes, or soluble cuticular lipids, embedded within the matrix, intracuticular waxes, or deposited on the outer surface of the plant cuticle, epicuticular waxes (*2*). In addition, significant amounts of polysaccharides and phenolics may also be present in the leaf and fruit cuticles (*1*). On the basis of their constituents, the cuticle can be defined as a hydrophobic and nonreactive polyester with associated waxes, the lipid complex composite more abundant in the biosphere.

The presence of phenolics, mainly cinnamic acids and flavonoids, in tomato fruit cuticles has been previously reported (*3*−*6*). Flavonoids are a class of phenolic compounds of low molecular weight widely distributed in the plant kingdom. These aromatic compounds exhibit a broad spectrum of biological functions and play an important role in the interaction between the plant and the environment (*7*). Using ^{3}H-phenylalanine as a precursor, it was shown that the flavonoid naringenin and its chalcone derivative, chalconaringenin, are synthesized in and transported from the epidermal cells to the waxes and cutin matrix of tomato fruit cuticle, where they are sorbed or deposited during fruit ripening (*8*). Previous studies have evidenced the role of flavonoids in the amorphous structure of the cuticular membrane, the ion-exchange capability, and the hydrodynamic properties of the cuticle. Thus, it has been reported that phenolics are responsible for most of the ionic exchange capacity of isolated fruit cuticles (*3*, *6*). Luque et al. (*6*) also reported that the flavonoids present in the cutin matrix of isolated tomato fruit cuticles play an important role in the water transport across the cuticle, being distributed into molecular clusters trapped in the amorphous polymeric hydrocarbon chains of the cutin. The authors also postulated that this molecular arrangement could have consequences for the mechanical expansion of the cuticle.

With regard to the rheological and mechanical properties, recently it was shown that the elastic modulus of the tomato fruit cutin, regardless of the temperature or hydration degree, was always significantly higher in the red ripe cuticles than in the mature green ones. On the other hand, the strain was lower, assuming that the presence of flavonoids in the cuticle network could explain the increase of rigidity from the mature green to the red ripe stage (*9*, *10*).

Due to their economical and nutritional relevance, flavonoids present in the tomato epidermis have recently become the subject of several studies and analyses. These concern both the analytical composition and metabolomic studies of flavonoids in many tomato fruit varieties (*11*, *12*). On the other hand, tomato fruit cuticle has been taken as a model to analyze the mechanisms of sorption of both polar and nonpolar organic compounds, mainly contaminants (*13*).

In the present work our goal has been to study in detail the interaction of the flavonoid naringenin (see its chemical structure in **Figure 1**) with isolated tomato fruit cuticles in two stages of development, mature green and red ripe, to gain insight into cuticle−flavonoid interactions, especially the characteristics of

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 Published on Web 07/27/2009

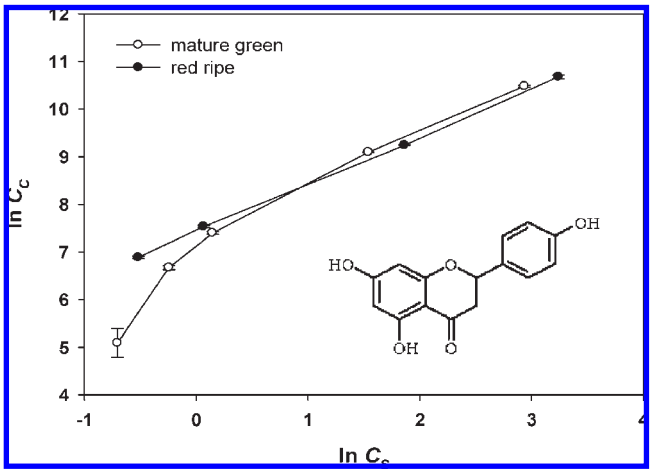

Figure 1. Sorption isotherms of naringenin to mature green (○) and red ripe (●) tomato fruit cuticles. The sorption process was carried out at 25 °C in the system cuticle/aqueous solution. The isotherm corresponding to red ripe cuticle was adjusted to the Freundlich equation ($r > 0.999$, $n = 1$, $k = 1675$). The lineal portion of the mature green cuticle isotherm also followed the Freundlich equation ($r > 0.99$, $n = 0.9$, $k = 1459$). Each point in the isotherms is the average of three replicates.

the flavonoid sorption and deposition. This objective has not been elucidated yet, this type of information being necessary for a better understanding of the different physiological and physical properties of the tomato fruit cuticle. Moreover, the present investigation could be used to expand the current information on the sorption of aromatic organic contaminants by plant cuticular materials.

MATERIALS AND METHODS

Cuticle Isolation. Cuticles were enzymatically isolated from tomato fruits *Solanum lycopersicum* L. cv. 'Caruso' at different stages of development following the protocol of Orgell (*14*) as modified by Yamada et al. (*15*) (see also ref *16*) using an aqueous solution of a mixture of fungal cellulase (0.2% w/v, Sigma, St. Louis, MO), pectinase (2.0% w/v, Sigma), and 1 mM NaN_3 to prevent microbial growth, in sodium citrate buffer (50 mM, pH 3.7). Vacuum was used to facilitate enzyme penetration, and fruit samples were incubated with continuous agitation at 35 °C for at least 14 days. The cuticle was then separated from the epidermis, rinsed in distilled water, and stored under dry conditions.

Partition Coefficient Determination. About 10 mg of enzymatically isolated cuticles from tomato fruits was cut with a sharp razor blade, and the pieces were placed in glass vials covered with Teflon-lined screws. Samples were equilibrated in 10 mL of 10 mM citrate buffer, pH 3.2, for 12 h in a shaker bath at 25 °C. Sodium azide (10 mM) was added to the solution to prevent fungal growth. Different naringenin (Sigma- Aldrich Chemical Co.) concentrations were added to each sample, and vials were placed in a shaker bath at a given temperature for 48 h, until the sorption equilibrium was reached. The role of naringenin concentration on the sorption process was studied at five different naringenin concentrations, 2.5×10^{-6}, 5×10^{-6}, 10^{-5}, 5×10^{-5}, and 2×10^{-4} M, in sodium citrate buffer, pH 3.2. Temperature remained constant at 25 °C during the experiments. The effect of temperature, 5, 15, 25, and 35 °C, on the sorption process was studied at a given naringenin concentration of 5×10^{-5} M.

The cuticle/water partition coefficient, K, for naringenin sorption was calculated according to the equation

$$K = C_c/C_s \quad (1)$$

where C_c is the concentration of the flavonid in the cuticle (g/kg) and C_s is the solute concentration (in the same units) of the flavonoid in the water solution at equilibrium. The concentration of naringenin sorbed on the solid sample was calculated as the difference between the initial concentration minus the concentration of the corresponding flavonoid sorbed at defined times.

Naringenin Determination. In each case, the concentration of naringenin was spectrophotometrically determined from the corresponding calibration curves at room temperature in citrate buffer. Naringenin concentration was measured at 288 nm.

Sorption Isotherm. A sorption isotherm relates the ln C_c with the ln C_s at a given temperature. The sorption of organic molecules to isolated cuticles has been tentatively explained using linear isotherms (*17*, *18*). An alternative to describe the physical sorption of molecules to heterogeneous sorbents, such as the tomato fruit cuticle, is the Freundlich equation

$$C_c = kC_s^{1/n} \quad (2)$$

or in the logarithmic form

$$\ln C_c = \ln k + (1/n)\ln C_s \quad (3)$$

where k and n are constants empirically related with the polymer ability to sorb and the intensity of this sorption, respectively (*19*). When $n = 1$, the sorption isotherm is linear and k equals the partition constant at any given concentration.

Thermodynamic Parameters. The change of Gibbs free energy due to the transfer of naringenin from an aqueous solution to the cuticle under constant pressure can be calculated according to

$$\Delta G^\circ = -RT \ln K \quad (4)$$

where R and T are the gas constant and the absolute temperature, respectively, and K is the partition coefficient above-described.

The transfer of naringenin from one phase to another involves changes in the enthalpy ($\Delta H°$) and entropy ($\Delta S°$). These parameters can be estimated from the corresponding plot of ln K versus T^{-1}, assuming that they are not a function of temperature within the temperature range considered (*19*):

$$\ln K = -(\Delta H^\circ/RT) + (\Delta S^\circ/R) \quad (5)$$

Statistics. Three replicates per naringenin concentration and temperature were conducted for each experiment of naringenin sorption to tomato fruit cuticles. Two aliquots per replicate were measured to estimate naringenin concentration. t tests were applied to compare means (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Flavonoid Sorption to Isolated Tomato Cuticle. Cuticle−chemical interactions are studied following physicochemical models of aqueous solute sorption to a solid sorbent represented by the cuticular membrane (*20*). The parameters most commonly used to quantitatively describe this sorption are the molar concentration of the solute and the partition coefficient (K), which can be defined as the solute concentration at equilibrium between the cuticle solid phase and the solution (*20*). This adimensional parameter is independent from the solute concentration.

Because the cuticle is mainly a lipophilic composite, it is expected for organic nonpolar compounds to have high partition coefficients. Partition coefficients of a wide variety of substances can be found in the literature (*20*−*23*). Moreover, some researchers have tried to correlate some molecular properties of the solute with experimental partition coefficients to find a model that could predict the solubility of a given compound in the cuticular membrane (*24*).

Despite the information available on partition coefficients of organic molecules and on the chemical composition of the cuticle, little is known on the physical structure and arrangement of cuticular components. As was indicated in the Introduction, the presence of flavonoids in some plant cuticles at certain stages of development provides a good model to investigate the nature of these solute−cuticle interactions, using naringenin as a molecular probe.

Table 1. Dependence of the Amount of Naringenin Sorbed by Mature Green Isolated Cuticles and Its Correspondent Partition Coefficient on the Time of Incubation[a]

time (h)	C_c (μg/g)	K
1	18.83 (0.79)	1142 (195);
2	19.66 (0.71)	1501 (259);
4	19.51 (0.38)	1422 (227);
7	19.73 (0.52)	1519 (166);
24	20.34 (0.39)	1956 (188);
32	20.25 (0.14)	1774 (91);

[a] Temperature remained constant at 25 °C. Initial naringenin concentration in the aqueous solution was 0.2 mM. Standard deviation corresponding to five replicates is included in parentheses.

Table 2. Partition Coefficients of Several Organic Compounds in the System Tomato Fruit Cuticle/Aqueous Solution Reported in the Literature (*24*)

organic compound	log K	organic compound	log K
phenol	1.58	2,4-dichlorophenoxyacetic acid	2.63
2-nitrophenol	1.83	2,4,5-trichlorophenoxyacetic acid	3.19
4-nitrophenol	1.89	pentaclorophenol	4.57
naphthaleneacetic acid	2.24	naringenin (present work)	3.27

Prior to the study of naringenin sorption to isolated tomato fruit cuticles, it is necessary to consider the dependence of naringenin sorption on the incubation time. **Table 1** shows these data obtained for naringenin sorption on mature green tomato fruit cuticles. Naringenin sorption is evaluated using two parameters: the amount of solute in micrograms per milligram of cuticle and the partition coefficient of the solute. Data indicated a fast sorption of naringenin, reaching equilibrium after 2 h. This sorption is very fast when compared with similar systems previously reported. In this sense, naphthaleneacetic acid sorption to tomato fruit and *Citrus* leaf cuticles reaches equilibrium after 24−48 h (*25*−*27*), and phenolics and nitrophenolics reach equilibrium after 48 h (*28*), whereas the cytokinine benzyladenine reached equilibrium only after 432 h (*29*).

The high partition coefficient obtained for the naringenin−cuticle system is particularly notable. **Table 2** shows some of the partition coefficients for isolated tomato fruit cuticles present in the literature. As can be observed, these partition coefficients vary between 38 and 3.1×10^6, and therefore the affinity of naringenin for the tomato cuticle is similar to that of hydrophobic organic compounds such as 2,4,5-trichlorophenoxyacetic acid. On the other hand, the partition coefficient of naringenin is considerably higher than that of organic acids such as naphthaleneacetic and 2,4-dichlorophenoxyacetic as well as organic compounds of similar nature such as 1-naphthol, phenol, 2-nitrophenol, and 4-nitrophenol. When the above results are taken into account, it seems clear that the polycyclic nature of naringenin significantly increases its affinity to the cuticle.

Naringenin Sorption Isotherms. The analysis of the effect of solute concentration on the sorption process provides more information on the flavonoid interaction with the cuticle. In this sense, a study of the sorption isotherm of naringenin in aqueous solution to isolated tomato fruit cuticles in two stages of development was performed. **Figure 1** shows the shape and characteristics of the corresponding isotherms. The log isothermal graphs indicated a remarkably different behavior for the cuticles at the mature green and red ripe stages. Thus, data from naringenin sorption to red ripe cuticles follow Freundlich's equation throughout the range of concentrations studied (**Figure 1**). The value obtained for the parameter n was almost 1, which indicates this is a "type C" or partition constant isotherm according to the classification proposed by Giles et al. (*30*). Naringenin sorption to mature green cuticles, on the other hand, did not follow the Freundlich equation in the range of concentrations studied. In this case, the concentration of naringenin sorbed (C_c) did not increase linearly (**Figure 1**) with the concentration of naringenin in the solution (C_s). A detailed analysis of the isotherm showed two lines with different slopes. The values for the parameter n (see eq 3) were very similar for both slopes: 0.45 for lower naringenin concentrations and 0.52 for higher ones. Hence, the process of naringenin sorption to the mature green cuticles is clearly dependent on the amount of flavonoid present in the aqueous solution.

Table 3. Dependence of the Partition Coefficient on the Concentration of Naringenin in the Aqueous Phase at a Given Temperature (25 °C)[a]

	K	
naringenin concentration (μM)	mature green	red ripe
2.5	355 (131);	
5	1012 (87);	1647 (132);
10	1407 (81);	1758 (128);
50	1911 (18);	1597 (20);
200	1902 (217);	1736 (224);

[a] Two tomato cuticle systems are studied: mature green and red ripe. Standard deviation corresponding to five replicates is included in parentheses.

As mentioned previously, it is possible to calculate the partition coefficient of naringenin in the system cuticle/aqueous solution from the isotherm data. **Table 3** shows the partition coefficients for cuticles at the mature green and red ripe stages as a function of the initial concentration of naringenin in solution. These values agree with the previous discussion on the type of isotherm that describes the flavonoid sorption to the cuticle. For red ripe cuticles the partition constant was independent of the concentration of naringenin and reached a mean value of 1684. On the other hand, for mature green cuticles, the partition constant increased with the amount of naringenin in solution, and only at high concentrations did K reach a plateau with a value around 1900. It is interesting to point out that the two values are not statistically different.

Naringenin sorption to red ripe cuticles can be classified as a lineal isotherm (**Figure 1**). Sorption of organic molecules such as naphthaleneacetic and 2,4-dichlorophenoxyacetic acids to red ripe tomato fruit cuticles shows a similar behavior (*13*, *23*, *27*, *29*). The two systems herein studied have in common the flexible nature of the substrate, which have regions of differential accessibility to the sorbent, the higher affinity of the solute for the substrate than for the solvent, and, finally, the solute ability to penetrate molecular regions of low accessibility (*30*, *31*).

Physical and Molecular Characteristics of Naringenin Sorption. With the characteristics above-mentioned taken into account, a time course of events at the molecular level during the naringenin sorption process to red ripe cuticles could be suggested. The initial step of naringenin sorption would be to penetrate the polymer in such a way that its incorporation to the sorbent would generate additional sorption sites proportional to the amount of solute sorbed. The model suggested by Giles et al. (*30*) gives the solid sorbent such a flexibility that for every molecule sorbed a new sorption site is created. In this way, a constant affinity of the sorbant for the sorbent is guaranteed. The flexible substrate would be in this case the cuticle polymer matrix, mainly constituted by interesterified 10,16-dihydroxyhexadecanoic acids, the main constituent of tomato fruit cutin (*6*). The polymer flexibility would be conditioned by its cross-linking degree and by the energy barrier of the glassy state described by our group in isolated tomato fruit cutins and cuticles of immature fruits (*32*). In this sense, it is interesting to note that the cuticle and cutin from

red ripe tomato fruits did not show any glass transition (*32*). This could be a consequence of the chemical annealing process in the polyester due to the accumulation of flavonoids into the molecular arrangement of the cutin polymer, thus contributing to the quenching of the glass transition temperature recorded at early stages of development.

Special emphasis should be put on how these pieces of evidence are connected to biomechanical data of isolated mature green and red ripe tomato cutin recently reported (*10*). We have postulated that the amount of flavonoids in the cutin network is correlated with a more rigid cutin matrix, restricting segmental mobility of the polyester chains and possibly reducing the free volume within the network, thereby increasing the overall matrix rigidity. This increase in rigidity would make the cutin matrix less elastic during ripening, as reflected by the low strain values of cutin from the red ripe samples.

Naringenin sorption and deposition on mature green cuticles could be explained in a different light. It should not be forgotten that these cuticles lack flavonoids. The partition constant of naringenin in the system mature green cuticle/aqueous solution increases with the amount of flavonoid in the range of (2.5−50($\times 10^{-6}$ M (**Figure 2**), implying that the flavonoid affinity changes during its interaction with the isolated cuticle sample. A similar behavior has not been described previously in the literature for the sorption of any chemical compound to isolated cuticles. Sorption properties at low concentrations of solute can be considered similar to that of a liquid sorbent where the solute−solute interactions are unlikely and the interactions between naringenin and specific functional groups of the cutin polymer predominate. As the concentration of solute increases, the solid nature of the membrane becomes more apparent and, contrary to what happens in liquids, only a limited volume is available for sorption. Additionally, the solute−solute interactions can become relevant enough to precipitate the solute, at high concentrations, inside the polymer matrix. That is, at high concentrations of flavonoids, chemical interactions between naringenin molecules are highly probable. These interactions would produce molecular aggregates, clusters, inside the polymer matrix.

The increase in the partition constant that takes place during naringenin incorporation to the mature green cuticles reached a limit from which the system behaved similarly to red ripe cuticles, namely, like a constant partition system. The amount of naringenin per unit of cuticle dry weight in the turning point between the two situations can be calculated from **Figure 2** and accounts for 2.38 mg of naringenin/g of cuticle, approximately. Above this amount, naringenin will be interacting mostly with other naringenin molecules and forming molecular clusters inside the polymer. These molecular clusters give special physical properties to the cuticles, as above-mentioned, and are responsible for the observed dependence of water permeability on pH and on the ionic nature of the membrane (*6*) together with the thermal and biomechanical properties above-described.

Thermodynamic Analysis of Naringenin Sorption to Tomato Cuticle. To complete the study of naringenin interaction with cuticular membranes, a thermodynamic analysis of naringenin partition between the cuticle and the aqueous solution was performed. This study can provide information on the molecular mechanisms of sorption and on the physical and chemical characteristics that rule this process of phase transference (*19*). **Table 4** shows the effect of temperature on naringenin sorption in mature green cuticles. A decrease in the partition coefficient can be observed as the temperature increased. Therefore, naringenin sorption can be regarded as an exergonic process, considering the negative change in free energy. The Gibbs equation (eq 4) allows an analysis of the change in free energy that happens during naringenin sorption and the calculation of the enthalpy and entropy change (eq 5). From **Figure 3**, values of −36.11 kJ/mol for the enthalpy and −59 J/K/mol for the entropy changes were obtained. Studies on the thermodynamics of the cuticle sorption can scarcely be found in the literature. The sorption of naphthalenacetic and 2,4-diclorophenoxyacetic acids to red ripe tomato cuticles produced a change in enthalpy of approximately −15 kJ/mol (*22*). For 4-nitrophenol, the change in enthalpy produced by the sorption to red ripe tomato cuticle was −20.5 kJ/mol (*17*) and −27.2 kJ/mol for *Ficus* cuticle (*17*). In all of these examples, as well as for the system here studied, the transference of a solute from an aqueous solution to the cuticle is a process enthalpically driven. Only in one known system,

Table 4. Temperature Dependence of the Partition Coefficient of the System Mature Green Tomato Cuticle/Naringenin[a]

temperature (K)	partition coefficient	temperature (K)	partition coefficient
278	4312 (404)	298	1911 (18);
288	3443 (273)	308	989 (45);

[a] The initial concentration of naringenin in the aqueous phase was 50 μM. Standard deviation corresponding to five replicates is included in parentheses.

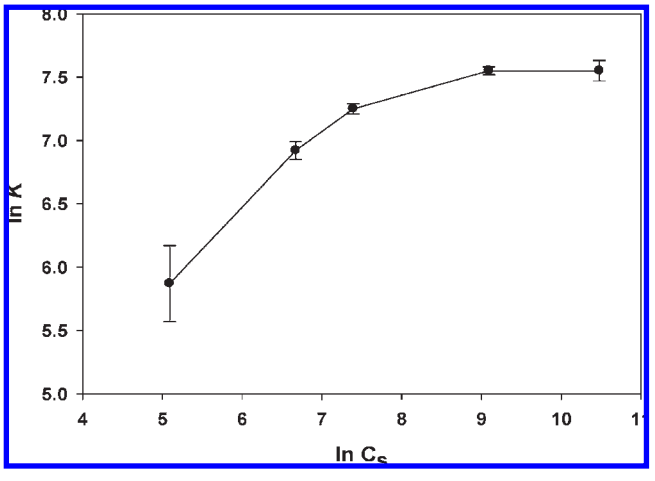

Figure 2. Partition coefficient (K) variation with the concentration of naringenin in the cuticle phase (C_c) at 25 °C in the system mature green tomato fruit cuticle/ aqueous solution.

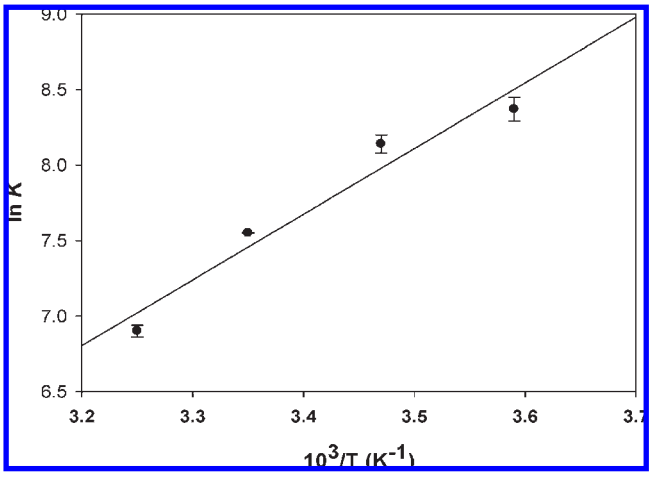

Figure 3. Temperature dependence of the partition coefficient of the system mature green tomato cuticle/naringenin. The concentration of naringenin in the aqueous phase was 50 μM.

methylene blue sorption to tomato fruit cuticle, was the process entropically driven (*22*).

The higher enthalpy change obtained in this research, compared to those described in the literature, probably reflects the existence of van der Waals interactions added to significant hydrogen-bonding interactions. Therefore, considering the above discussion, most of the total energy would be assigned to the interaction between naringenin molecules, which would cluster between the hydrocarbon chains of the cutin. Nevertheless, the formation of hydrogen bonding between the hydroxyl groups of the naringenin and between these hydroxyl groups and polar groups of the cuticle matrix, probably ester functional groups, cannot be ruled out.

In summary, this study demonstrates the role of some endogenous aromatic components, flavonoids, present in the cuticle of ripening tomato fruits, in the control of the sorption capacity of the polymer matrix, modeling thus their thermal and mechanical properties. At the same time, this study would add to a better understanding of the sorption characteristics of plant cuticular materials.

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Received April 6, 2009. Revised manuscript received June 13, 2009. Accepted July 14, 2009. This project was supported by Research Grant AGL2006-12494 from Plan Nacional de I+D, Ministerio de Educación y Ciencia, Spain.