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Enzymatic Acylation of Flavonoids: Effect of the Nature of the Substrate, Origin of Lipase, and Operating Conditions on Conversion Yield and Regioselectivity

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The conversion yield at equilibrium, the initial rate, and the regioselectivity of the enzymatic acetylation of aglycone flavonoids (quercetin, naringenin, hesperetin, and chrysin) were investigated and compared to those obtained with a glycosylated one (isoquercitrin). The effects of a wide range of operating conditions were quantified. Fourier transform infrared spectrometry (FT-IR), NMR, and high performance liquid chromatography electrospray ionization mass spectrometry (HPLC–ESI–MS) analyses showed that for glycosylated flavonoids, in the presence of *Candida antarctica* (CAL-B), the acetylation occurred on the 2″-OH, 3″-OH, and 6″-OH of the glucose part, while with *Pseudomonas cepacea* lipase (PSL-C) acetylation takes place on 6″-OH of the sugar and 4′-OH of the B-ring. For aglycone flavonoids, the acetylation occurred only with PSL-C on 4′-OH, 3′-OH, and 7-OH hydroxyls. The conversion yield and the number and the relative proportions of the synthesized products were found dependent on the nature of the enzyme, the molar ratio, and the flavonoid structure. The initial rate was affected only by the origin of the enzyme.

KEYWORDS: Flavonoid; acetylation; *Candida antarctica* lipase B; *Pseudomonas cepacia* lipase C; regioselectivity

INTRODUCTION

Flavonoids are a large group of phenolic plant constituents. To date, more than 6000 flavonoids have been identified. Essentially, two forms of flavonoids are present in plants: aglycone and glycosylated. Compared to the glycosylated forms, the aglycones show a high biological activity in vitro (*1–4*).

In recent years, there has been a growing academic and industrial interest in the health benefits of flavonoids. It originates from observations that increased consumption of fruits and vegetables may be an important factor in the prevention of proliferative diseases such as cancer, coronary heart disease, and neurodegenerative diseases. Flavonoids have been observed to have biological in vitro effects, such as free radical scavenging, modulation of enzymatic activity, and inhibition of cellular proliferation, as well as to demonstrate activities as antibiotic, antiallergic, antidiarrheal, antiulcer, and anti-inflammatory agents (*5–7*).

The possible beneficial effects of flavonoids have resulted in their use as food supplements. A variety of flavonoid extracts from plants are available on the market. Recently, extracts of berries, grapes, pine barks, containing mainly flavonols and anthocyans, have been marketed by ingredients companies and used in beverages and food. Many of the large food companies have interests in flavonoids to meet consumers' demand for healthier food. Thus, flavonoids may carry many opportunities of food ingredient innovations and business developments. However, depending on their structure, the processability of these compounds is still limited by their weak stability and low solubility in organic or aqueous solvents (*8–10*). With a view to improving these properties, derivatization of flavonoids by glycosylation and acylation were reported in several works (*9, 11–14*). These reactions can be carried out by chemical, enzymatic, or chemoenzymatic methods. Because of the numerous reactive hydroxy groups in flavonoid structures, the use of chemical methods requires many protection/deprotection steps to obtain selective functionalization. For this reason, the enzymatic route, which is more regioselective, was described as promising. The main studies dealing with flavonoid acylation were carried out with glycosylated forms using *Candida antarctica* lipase (CAL-

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B) as a biocatalyst (*8, 11, 14–23*). For aglycone flavonoids, only two studies were reported (*12, 13*).

The propensity of a flavonoid biological activities is governed by its chemical structure. In fact, it has observed that the lack of the C2–C3 double bound, the C-3 hydroxy group, and the 3′,4′ dihydroxy (or prenyl) on the B-ring of the flavonoid is the origin of the decrease of their biological activities. So, to preserve the beneficial effects of flavonoids in food applications, great attention must be given to the number, positions, and types of substitutions occurring during the acylation reaction. This fact is more critical for aglycone flavonoids because the substitution takes place only on the hydroxy groups of A, B, and C rings, while for glycosylated flavonoids the acylation occurs on the glycosylated part (*11, 14*).

The aim of this work was to investigate the effect of several factors (solvent, substrates molar ratio, enzyme origin, and flavonoid structure) on the regioselectivity and conversion yield of enzymatic acylation of flavonoids by lipases. This knowledge is needed to select the operating conditions allowing the synthesis of flavonoid derivatives with improved activities for food applications.

MATERIALS AND METHODS

Enzymes and Chemicals. Immobilized CAL-B (Novozym 435, acrylic resin, EC 3.1.1.3, 7000 PLU/g: propyl laurate units synthesized per gram of catalyst) and immobilized PSL (PSL-C II, ceramic particles, 752 U/g: one unit will convert 1 μmol of 1-phenethyl alcohol to 1-phenethyl acetate per min at 25 °C in the presence of vinyl acetate) were purchased from Novo Industries and Amano Enzyme Inc., respectively.

The flavonoids tested were quercetin (purity 98%, Sigma), isoquercitrin (purity 95%, Extrasynthese), naringenin (≥95% purity, Sigma), hesperetin (≥98% purity, Sigma), and chrysin (≥95% purity, Acros). Palmitic acid, vinyl acetate, vinyl propionate, vinyl butyrate, and vinyl palmitate (purity 99%, Aldrich) were used as acyl donors. The acetylation reactions were performed in acetonitrile (purity 98%, Merck), acetone (purity 98%, Carlo Erba Reactifs), or *tert*-amyl alcohol (purity 99%, Carlo Erba Reactifs).

Flavonoid Ester Synthesis Procedure. The enzymatic synthesis of flavonoid esters was performed in an ASW 1000 automated synthesis workstation (Chemspeed, Augst, Switzerland) equipped with microreactors of 27 mL capacity with a working volume kept constant to 15 mL. Before each experiment, solvent and acyl donor were dried on 4 Å molecular sieves and added to the flavonoid (10 g/L). Acyl donor was used in excess and the molar ratio acyl donor/flavonoid was varied from 5 to 40. The mixture was stirred for 12 h at 50 °C. The transesterification reaction was started by adding 50 g/L of immobilized lipases [(CAL-B or *Pseudomonas cepacea* lipase (PSL-C)]. Samples were taken from the reaction medium at different times, diluted in the same solvent used for biotransformation, and analyzed by HPLC. Control experiments without enzymes were also carried out, and no reactivity was observed. The reactions lasted for 7 days.

The water content of the medium was measured at the beginning and the end of the reaction (0.15%, w/w) with a KF737II coulometric Karl Fisher apparatus. Hydranal-coulomat AG-H (Sigma) was used as a reagent.

HPLC Analytical Procedure. The transesterification reaction of flavonoids was monitored by HPLC. Analyses were carried out in a Lachrom system (Merck KGaA, Darmstadt, Germany) equipped with a 250 × 4.6 mm i.d. Apollo C18 column (Alltech, Templemars, France), a L-7350 column oven (Merck KGaA, Darmstadt, Germany), an L-7200 auto-injector (Merck KGaA, Darmstadt, Germany), and a UV detector (Merck KGaA, Darmstadt, Germany) maintained at 245 nm. The different compounds were separated using a methanol (A)/water (B) elution system. A three step gradient was applied: 0 min (40% A), 5 min (100% A), 10 min (100% A), and 15 min (40% A). Elution flow rate was 1 mL/min. The elution was performed at 55 °C.

Calibration curves for the flavonoids were obtained using standards in acetonitrile, acetone, or *tert*-amyl alcohol, depending on the reaction–solvent.

The conversion yields were expressed by the molar ratio of consumed to initial flavonoid. The relative proportions of flavonoid esters were determined by the ratio of the peak area of each ester to the total area of products. Reaction rates were calculated from the slope of the linear portion of plots of the decrease of flavonoid concentration versus time, and expressed as mM/h of biocatalyst.

FT-IR Analysis. IR spectra were recorded on a Perkin-Elmer Fourier transform infrared spectrometry (FT-IR) (Courtaboeuf, France) in attenuated total reflection (ATR) mode.

The FT-IR analyses of the synthesized esters were compared to those of substrates. The first observation was the appearance of a C–O stretching band (1720 cm^{-1}), corresponding to ester functions. The second one was a diminution of the intensity of the O–H stretching band (3300 cm^{-1}), assigned to hydroxy acetylation. The magnitude of this variation is accentuated when the reaction takes place on phenolic hydroxy groups.

NMR and HPLC–ESI–MS Analytical Procedure. The residual flavonoids and flavonoid esters were purified by preparative high performance liquid chromatography (HPLC). A 25 × 5 cm i.d., 12 μm C18 column (Alltech, Templemars, France) was used. The chromatographic separation was carried out using a linear gradient from 20 to 100% methanol in water over 30 min. The flow-rate was maintained at 20 mL/min throughout the run. Elution was performed at ambient temperature with UV detection.

The chemical structure of flavonoids and their acetylated products, obtained with a high purity, was determined by ^{1}H NMR and ^{13}C NMR in DMSO-d_6 using a Bruker AM 400 spectrometer (Bruker, courtaboeuf, France) at 400.13 and 100.58 MHz, respectively. The coupling constant J was expressed in hertz. Tetramethylsilane was used as an internal reference. The ^{13}C NMR analysis was carried out overnight for better signal/noise ratio.

For high performance liquid chromatography electrospray ionization mass spectrometry (HPLC–ESI–MS) analysis, chromatography was performed on a 9xx Jasco system (Jasco, Bouguenais, France), equipped with a UV-975 detector (Jasco, Bouguenais, France), coupled to a VG Platform II mass spectrometer (Frisons Instruments, Arcueil, France) equipped with an electrospray source operating in both positive and negative ionization modes. The source temperature was set at 80 °C with a cone voltage of 40 V. A 10 μL aliquot sample was injected into a 250 × 4.6 mm i.d. Apollo C18 column (Alltech, Templemars, France). The column was eluted with methanol (A)/water (B): 0 min (40% A), 5 min (100% A), 10 min (100% A), and 15 min (40% A). At the end of the column flow was split with 95% used for UV detection and 5% directed towards the mass spectrometer. The mass spectrometric data were collected in full scan mode from m/z 100 to 600, from 0 to 17 min.

RESULTS AND DISCUSSION

Several acyl donors (palmitic acid, vinyl acetate, vinyl propionate, vinyl butyrate, vinyl palmitate) were tested for the acylation of **1** with PSL-C. Only vinyl esters were reactive. Vinyl acetate was chosen to investigate in depth the effect of several factors (origin of the lipase, nature of the solvent, molar ration of substrates and nature of the flavonoid) on the performance and the regioselectivity of the acylation reaction.

Effect of the Origin of the Lipase. *Effect on Conversion Yield and Initial Rate.* The enzymatic acetylation of quercetin (**1**) and isoquercitrin (**2**) by vinyl acetate was studied using two enzymes, either CAL-B or PSL-C, in acetone, at 50 °C. The molar ratio of vinyl acetate to flavonoid was 40. These two substrates have the same aglycone part (**Figure 1**). Isoquercitrin is a glycosylated form of quercetin; the sugar moiety is a glucose linked at position 3 (quercetin 3-β-D-glucoside). Both CAL-B and PSL-C catalyzed the acetylation of isoquercitrin, while only PSL-C was active with quercetin.

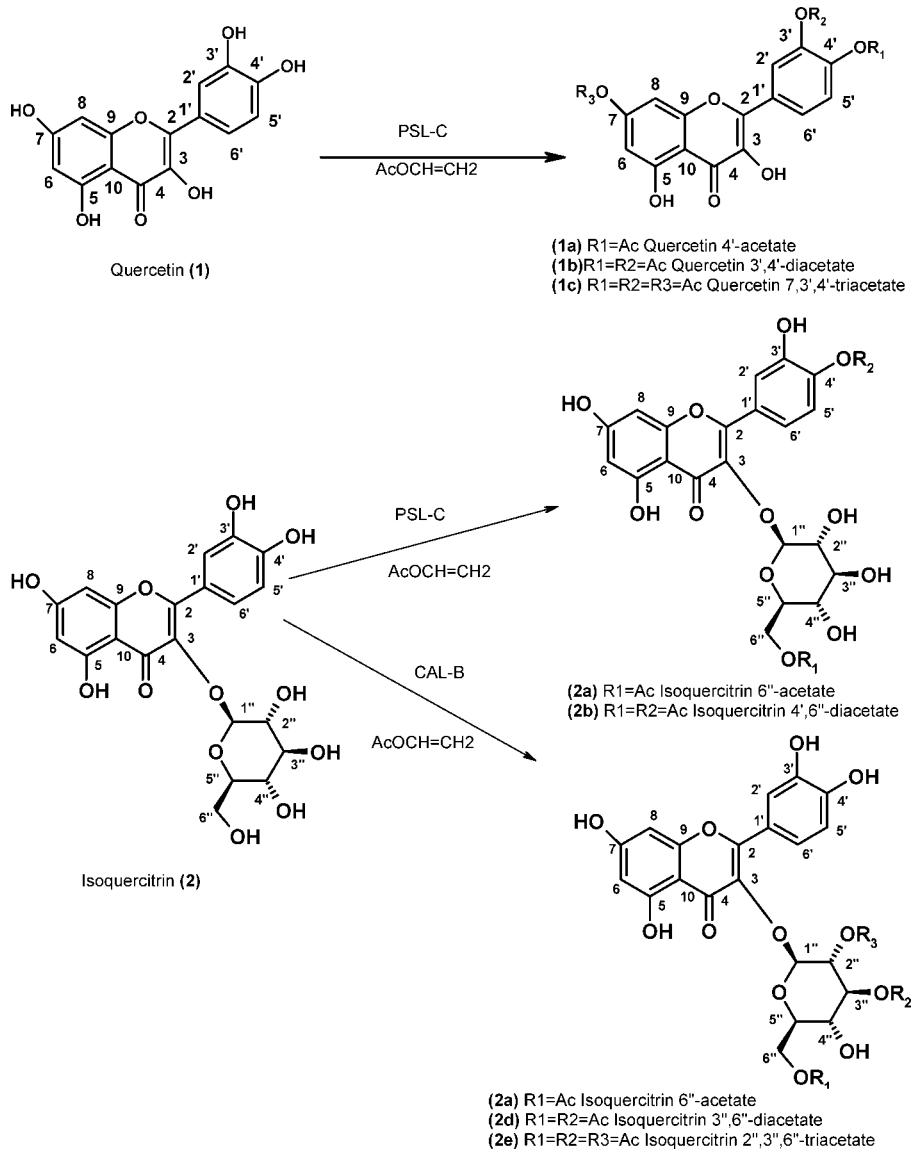


Figure 1. Enzymatic acetylation of quercetin (**1**) and isoquercitrin (**2**) catalyzed by PSL-C and CAL-B in acetonitrile, acetone, and *tert*-amyl alcohol using vinyl acetate as acyl donor.

During the acetylation of isoquercitrin by PSL-C in acetone (**Figure 2A**), the concentration of the starting material decreased rapidly, and it was almost all consumed after 24 h of incubation. The final conversion yield of isoquercitrin and the initial rate were 100% and 4.9 mM/h, respectively. Three products P1, P2, and P3 were synthesized. P1 was produced at the early stage of the reaction. After 2 h of incubation, P1 concentration decreased leading to the formation of P2 and P3. Similar profiles were mentioned by Min et al. (*24*) during the synthesis of fructose esters with *Pseudomonas* sp.

For the acetylation of isoquercitrin (**2**) with CAL-B (**Figure 2B**), the HPLC analysis indicated a conversion yield of 100% after 96 h of incubation and the formation of only two products. The initial rate of this reaction (8 mM/h) was double that obtained with PSL-C.

As previously mentioned, only PSL-C catalyzed the acetylation of the aglycon form of isoquercitrin (quercetin, **Figure 3**). This reaction led to the formation of three products. P1 was synthesized in the first step of the reaction, then the formation of P2 and P3 was observed. The initial rate and the flavonoid conversion yield after 96 h of incubation were 4 mM/h and 84%, respectively. The comparison of these results to those obtained with isoquercitrin showed that the reaction occurred with a similar initial rate and led to a similar number of compounds produced. However, the conversion yield of the glycosylated substrate was slightly higher than the aglycone form.

Effect on Regioselectivity. The comparison of FT-IR spectra of quercetin (**1**) and compounds produced showed a significant diminution in the signal at 3300 cm^{-1}. This behavior could be due to the multiple acetylation on the phenolic ring of the quercetin. HPLC–ESI–MS analysis allowed the identification of P1 (**1a**) as a monoacetate [M – H^-, *m/z* 343], P2 (**1b**) as a diacetate [M – H^-, *m/z* 385], and P3 (**1c**) as a triacetate [M – H^-, *m/z* 427] of quercetin (**Figure 3**).

^1H and ^{13}C NMR characterization of these esters showed that the three products obtained were successively quercetin 4′-acetate (**1a**), quercetin 3′,4′-diacetate (**1b**), and quercetin 7,3′,4′-triacetate (**1c**) (**Figure 3**). The location of the acetylated sites was determined by comparing upfield and downfield shifts between products and quercetin. ^{13}C NMR spectra showed that the acetylation on the 4′-OH position led to an upfield shift of the C4′ signal of −9 ppm and a downfield shift of the C3′ and C5′ signals (ortho position) of 6 ppm. These shifts are due to the mesomeric effect (*25*). Moreover, ^1H NMR analysis indicated a downfield shift of the adjacent proton signal (5′-H) confirming the acetylation on the 4′-OH.

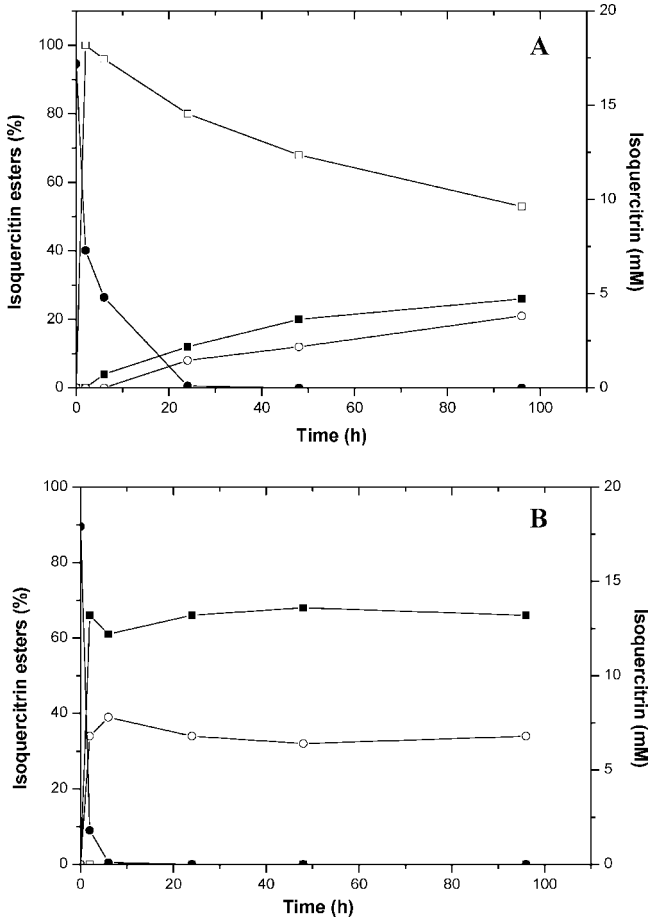


Figure 2. (**A**) Reaction progress of isoquercitrin (20 mM) acetylation with vinyl acetate (800 mM) catalyzed by PSL-C in acetone at 50 °C; (•) isoquercitrin, (□) P1 product, (■) P2 product, and (○) P3 product. (**B**) Reaction progress of isoquercitrin (20 mM) acetylation with vinyl acetate (800 mM) catalyzed by CAL-B in acetone at 50 °C; (•) isoquercitrin, (■) P1 product, and (○) P2 product.

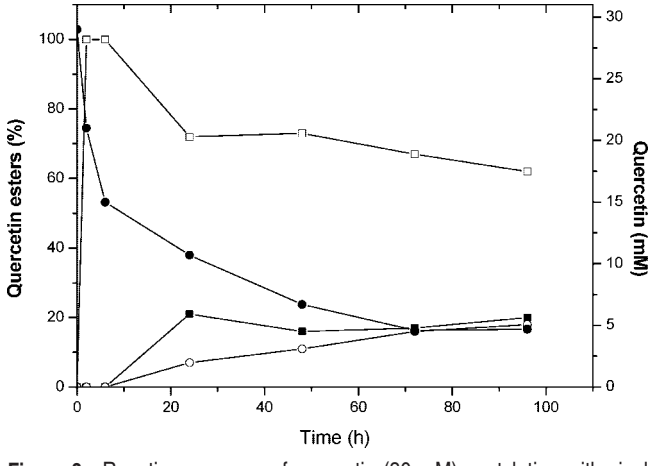


Figure 3. Reaction progress of quercetin (30 mM) acetylation with vinyl acetate (1200 mM) prepared by PSL-C catalyzed in acetone at 50 °C; (•) quercetin, (□) P1 product, (■) P2 product, and (○) P3 product.

For isoquercitrin (**2**), FT-IR analysis indicated a less pronounced diminution of the signal located at 3300 cm^{-1} suggesting a lower degree of acetylation on the phenolic ring. HPLC–ESI–MS analysis of the products, obtained after 96 h of incubation with PSL-C, showed that the masses of P1 (**2a**), P2 (**2b**), and P3 (**2c**) were [M – H^-, *m/z* 505, 547, and 589], respectively (**Figure 2A**). ^1H and ^{13}C NMR analyses revealed that **2a** and **2b** were isoquercitrin 6″-acetate and isoquercitrin 4′,6″-diacetate, respectively. The structure of the triester **2c** has not yet been elucidated due to its low concentration. As previously described, acetylation sites were located by comparing the signal shifts between isoquercitrin and the products ^1H and ^{13}C NMR spectra. The acetylation on the glycosidic moiety showed a downfield shift of the saturated carbons signal of 2.72 ppm and an upfield shift of the adjacent carbons signal of 3.87 ppm. Moreover, ^1H NMR analysis showed that both the signals of H-6″a and H-6″b were shifted downfield by 0.8 ppm, and the signals of H-2′ and H-5′ were shifted downfield by 0.4 and 0.2 ppm, respectively. The C6″ signal shifts of isoquercitrin 4′,6″-diacetate (**2b**) were similar to those described previously for isoquercitrin 6″-acetate. The C4′ signal was shifted upfield (10.7 ppm), whereas C3′ and C5′ signals were shifted downfield (7.06 and 4.5 ppm, respectively).

These results suggested that the regioselectivity of the reaction depends mainly on the class of available hydroxy groups (primary, secondary, phenolic) and their position. When a primary hydroxy group is present (isoquercitrin), acetylation first takes place at this position. This result was previously reported and was explained by the high reactivity and the accessibility of this functional group. When only phenolic hydroxy groups are available, quercetin acetylation occurs successively on the 4′-OH, 3′-OH, and 7-OH positions.

The elucidation of the structures of the two products obtained during the acetylation of isoquercitrin by CAL-B was also investigated by FTIR, HPLC–ESI–MS, and NMR analyses. In FT-IR spectra, no variation of intensity of the signal at 3300 cm^{-1} was observed, indicating that the acetylation occurred only on the sugar part. The masses obtained by HPLC–ESI–MS analysis indicated that P1 (**2d**) and P2 (**2e**) were isoquercitrin diacetate [M – H^-, *m/z* 547] and isoquercitrin triacetate [M – H^-, *m/z* 589], respectively (**Figure 2**B). ^1H and ^{13}C NMR spectra allowed the identification of these products as isoquercitrin 3″,6″-diacetate (**2d**) and isoquercitrin 2″,3″,6″-triacetate (**2e**). In the ^{13}C NMR spectum of isoquercitrin 3″,6″-diacetate, the signals of C-3″ were shifted downfield by 0.6 ppm, whereas C-2″ and C-4″ signals were shifted upfield by 2.4 and 2.13 ppm, respectively. These data were confirmed by the ^1H NMR spectrum. The α H-3″ signal was shifted downfield by 1.57 ppm, while the β H-2″ and H4″ signals were shifted downfield by 0.3 ppm.

The production of isoquercitrin 3″,6″-diacetate (**2d**) is in accordance with the results reported by Danieli et al. (*18*). However, the presence of isoquercitrin 2″,3″,6″-triacetate (**2e**) was not mentioned by these authors. This difference can be explained by the fact that the molar ratio acyl donor/flavonoid was 10, while in our work it was 40.

Contrary to Nakajima et al. (*23*) and Stevenson et al. (*26*) who indicated that the acylation of isoquercitrin with vinyl cinnamate and 2-hydroxy-phenylpropionic acid, respectively, led to the synthesis of only isoquercitrin 6″-ester, no isoquercitrin 6″-ester was observed in this work.

Effect of the Nature of the Solvent. *Effect on Conversion Yield and Initial Rate.* The enzymatic acetylation of glycosylated flavonoids and their aglycone forms, such as isoquercitrin (**2**) and quercetin (**1**), was investigated in acetonitrile, acetone, and *tert*-amyl alcohol using PSL-C as a biocatalyst. The results obtained (conversion yield, initial rate) are summarized in **Table 1**. The comparison of these data indicated that the influence of the nature of the solvent depends on the nature of the flavonoid. For isoquercitrin, the nature of the solvent affected neither the conversion yield (100%) nor the initial rate (4–4.9 mM/h), while

Table 1. Initial Rate and Conversion Yield (96 h of incubation) for the Acetylation of Quercetin and Isoquercitrin with Vinyl Acetate Catalyzed by PSL-C in Acetonitrile, Acetone, and *tert*-Amyl Alcohol[a]

	initial rate (mM/h)		conversion yield (%)	
solvent	quercetin	isoquercitrin	quercetin	isoquercitrin
acetonitrile	4 ± 0.32	n.d.	96 ± 7.68	100 ± 8
acetone	4 ± 0.32	4.9 ± 0.39	84 ± 6.72	99 ± 7.72
tert-amyl alcohol	4.9 ± 0.39	4 ± 0.32	68 ± 5.44	98 ± 7.84

[a] Data are expressed as mean ± standard deviation ($n = 3$). n.d.: not determined due to the heterogeneity of the medium and sampling difficulties.

Table 2. Effect of the Acyl Donor/Quercetin Molar Ratio on the Reaction Rate, the Conversion Yield (96 h), and the Fractions of Products of the Acetylation of Quercetin (30 mM) with Vinyl Acetate Catalyzed by PSL-C in Acetonitrile

molar ratio	initial reaction rate (mM/h)	conversion yield (%)	flavonoid esters produced (%)		
			monoacetate	diacetate	triacetate
5	0.16	55	63	37	n.d.[a]
10	0.5	88	43	50	7
20	1	96	43	51	6
40	4	96	31	46	23

[a] n.d. = not detected.

for quercetin the nature of the solvent affected the conversion yield. Acetonitrile led to the highest conversion yield (96%). These results are in accordance with those obtained by Lambusta et al. (*12*), who studied the effect of several solvents (acetonitrile, tetrahydrofuran, *tert*-amyl alcohol, acetone) on catechin acetylation using PSL. They observed that acetonitrile led to the highest conversion yield. The differences in solvent effects can be explained by several factors (Log P, dielectric constant (ε), solubility, hydrophobicity). In fact, the data reported by Hazarika et al. (*27*) showed that Log P and dielectric constant are the main determining factors. They indicated that a high conversion yield is favored by a solvent with a low Log P and a high dielectric constant. These observations are in accordance with data obtained in this work.

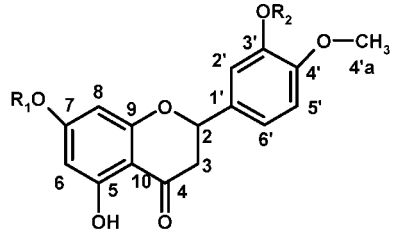
These results can be also explained by the difference of the flavonoid solubility in the solvents used. In acetonitrile, the solubility of quercetin was found to be only 6 mM. In the presence of the other two solvents, up to 30 mM of this compound was soluble. Moreover, in the case of acetonitrile, precipitation of products was observed that may shift the equilibrium in favor of ester synthesis.

Effect on Regioselectivity. For the enzymatic acetylation of **1** and **2** in acetonitrile, *tert*-amyl alcohol, and acetone three products were obtained: mono-, di-, and triacetates. In all cases, the formation of P2 and P3 was associated with the depletion of P1.

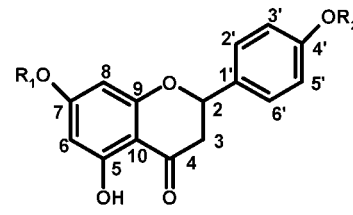
The molecular weights and the structures of the products were determined by HPLC–ESI–MS and ^{1}H, ^{13}C NMR analyses, respectively. No effect on the regioselectivity of the acetylation was observed whatever the solvent used. However, the percentages of mono-, di-, and triacetates, after 96 h of incubation, differed with the nature of the flavonoid and the solvent. With isoquercitrin, in acetone and *tert*-amyl alcohol, P1 was the major product (60 and 80%, respectively), while in acetonitrile P2 was the major product (50%). Similar behavior was observed with quercetin.

These results are in accordance with those obtained by McManus et al. (*28*) and Lopez et al. (*29*), for the acetylation, with PSL, of 6-*O*-trityl-β-D-glucopyranose and alkyl β-D-xylopyranosides, respectively. They reported that the relative ratio between the products was dependent on the polarity of the organic solvent.

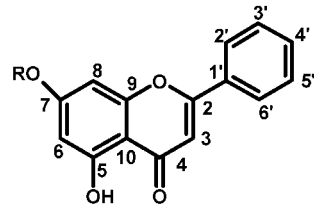
Effect of the Molar Ratio of Substrates. *Effect on Conversion Yield and Initial Rate.* The effect of the molar ratio of substrates was investigated in reaction media containing 30 mM of quercetin (**1**) and PSL-C as biocatalyst. The molar ratio was increased from 5 to 40. The quantification of this effect was evaluated by determining the initial rate and the final conversion yield (96 h) of the reaction. The results are summarized in **Table 2**. They show that both the initial rate and the conversion yield were affected by the molar ratio of substrates. The conversion yield and the initial rate increased when the molar ratio vinyl acetate/quercetin varied from 5 to 40. Similar behavior was reported for the enzymatic acylation of various flavonoids in organic media (*20, 22, 30*) and ionic liquids (*19*). The influence of the molar ratio of substrates on the conversion yield could be attributed to the thermodynamic shift of the equilibrium to the synthesis of quercetin acetates due to the vinyl acetate excess.



(3) R1=R2=H Hesperitin
(3a) R1=Ac R2=H Hesperitin 7-acetate
(3b) R2=Ac R1=H Hesperitin 3'-acetate



(4) R1=R2=H Naringenin
(4a) R2=Ac R1=H Naringenin 4'-acetate
(4b) R2=Ac R1=Ac Naringenin 7,4'-diacetate



(5) R=H Chrysin
(5a) R=Ac Chrysin 7-acetate

Figure 4. Structure of the hesperetin, chrysin, naringenin, and their acetates synthesized by PSL-C in acetonitrile using vinyl acetate as acyl donor.

Effect on Regioselectivity. The effect of the molar ratio of substrates on the regioselectivity of the reaction is summarized in **Table 2**. For a molar ratio of 5, only mono- (63%) and diester (37%) were synthesized. For a higher molar ratio (10, 20, 40), mono-, di-, and triacetates were formed. Moreover, higher molar ratios favored the synthesis of triester. For PSL-C, to our knowledge, no data are available in the literature about the effect of the acyl donor/flavonoid molar ratio on the selectivity. For CAL-B, several studies (*31, 32*) reported that the formation of triester did not lead to a decrease in monoester.

Effect of the Nature of the Flavonoid. The effect of flavonoid structure on the regioselectivity of the acetylation catalyzed by PSL-C was studied by using hesperetin (**3**), naringenin (**4**), and chrysin (**5**) in acetonitrile, at 50 °C. Similarly to **1**, the acetylation yields at 96 h, were almost 100%, for naringenin, but only 30 and 15% for hesperetin and chrysin, respectively. The number of synthesized products depended on the nature of the flavonoid. For hesperetin and naringenin, two products were synthesized, while with chrysin only one product was observed.

Structural determination of the products (HPLC–ESI–MS, and NMR analyses) allowed the acetylated positions to be located (**Figure 4**). In the case of naringenin, the two products were naringenin 4′-acetate (**4a**) [M – H$^-$, *m*/*z* 313] and naringenin 7,4′-diacetate (**4b**) [M – H$^-$, *m*/*z* 355]. With hesperetin, the two isomeric monoesters detected were hesperetin 7-acetate (**3a**) and hesperetin 3′-acetate (**3b**) [M – H$^-$, *m*/*z* 343], while with chrysin only chrysin 7-acetate (**5a**) [M – H$^-$, *m*/*z* 295] was synthesized. For quercetin, as previously shown, the acetylation took place on the 3′,4′- and 7-OH groups. These results confirm that only PSL-C is able to acylate phenolic groups, as was described by Lambusta et al. (*12*) when using catechin as substrate.

The reactivity of the 4′-OH group of quercetin was also reported by Van Acker et al. (*33*), Russo et al. (*34*), and Erkoc et al. (*35*) using ab initio (STO-3G) and semiempirical (AM1 and PM3) methods. These authors also indicated the presence of an intramolecular H-bond: H5-O4-H3. Thus, the engagement of the 3-OH and 5-OH groups in the intramolecular H-bond probably explains the absence of acylation in these positions in our study.

In the absence of a 4′-OH group (hesperetin), PSL-C shows a preference for the 7-OH group, followed by the 3′-OH. The low reactivity of the 3′-OH group of hesperetin compared to quercetin may be explained by steric hinderance from the methoxy group at C-4′.

Finally, with chrysin, that has only two hydroxy groups at the 5-OH and 7-OH positions, the acylation took place on the 7-OH. This result confirms that the 5-OH group is not reactive when a 4-oxo group is present in the structure of the flavonoid. The reactivity of the 7-OH group was observed by Shin et al. (*36*) during chemical acylation of chrysin. However, in the absence of the 4-oxo group (catechin), Lambusta et al. (*12*) reported that the acetylation of catechin by PSL occurred on both the 5 and the 7-OH groups.

In conclusion, our data showed that for aglycone flavonoids the acylation takes place on 3′,4′-dihyroxy of B ring. This fact could lead to a decrease of the biological activities. So, for their use in food application it is necessary to evaluate the biological activities of these derivatives.

Supporting Information Available: Molecular weights and NMR characterizations of the used substrates (flavonoids) and the obtained derivatives (products). This material is available free of charge via the Internet at http://pubs.acs.org.

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