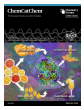

Covalent Immobilization of Naringinase over Two-Dimensional 2D Zeolites and its Applications in a Continuous Process to Produce Citrus Flavonoids and for Debittering of Juices

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The crude naringinase from *Penicillium decumbens* and a purified naringinase with high α-L-rhamnosidase activity could be covalently immobilized on two-dimensional zeolite ITQ-2 after surface modification with glutaraldehyde. The influence of pH and temperature on the enzyme activity (in free and immobilized forms) as well as the thermal stability were determined using the specific substrate: *p*-nitrophenyl-alpha-L-rhamnopyranoside (Rha-pNP). The crude and purified naringinase supported on ITQ-2 were applied in the hydrolysis of naringin, giving the flavonoids naringenin and prunin respectively with a conversion >90% and excellent selectivity. The supported enzymes showed long term stability, being possible to perform up to 25 consecutive cycles without loss of activity, showing its high potential to produce the valuable citrus flavonoids prunin and naringenin. We have also succeeded in the application of the immobilized crude naringinase on ITQ-2 for debittering grapefruit juices in a continuous process that was maintained operating for 300 h, with excellent results.

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Naringinase is an enzyme complex consisting of α-L-rhamnosidase (EC 3.2.1.40) and β-glucosidase activities (EC 3.2.1.21) widely applied in the food and pharmaceutical industries because of its capacity to hydrolyze many glycosides, e.g., 6-O-α-L-rhamnopyranosyl-β-D-glucopyranosides, naringin, hesperidin, and rutin.[1,2] Particularly, naringin (4′,5,7-trihydroxyflavone-7-rhamnoglucoside) is a glycosylated flavonoid highly present in peel, membrane and seed, being the main bitter component in citrus juices. Naringin can be hydrolyzed into non-bitter components by the enzyme nariginase. Thus, the α-L-rhamnosidase hydrolyzes the naringin into rhamnose and prunin (which is one-third as bitter as naringin), while in a subsequent step the glucosidase hydrolyzes prunin into glucose and aglycone naringenin, which are non-bitter components (see Scheme 1).[1,3]

The hydrolysis products, the monoglycoside flavonoid, prunin (4′,5,7-trihydroxyflavanone-β-D-glucoside), and the aglycone naringenin (4′,5,7-trihydroxyflavanone) are key compounds with a great potential in the pharmaceutical, cosmetic and food industries due to a varied range of biological activities such as anticancer, anti-inflammatory, antioxidant, antiulcer, antiviral and neuroprotective activity.[3,4] However, the synthesis of naringenin and particularly the monoglycoside flavonoid, prunin by conventional chemical methods is not an easy task because the existence of side reactions that lead to low selectivity to the target compound. Therefore, the production of prunin and naringenin through the hydrolysis of naringin (which can be easily extracted from citrus wastes) using enzymatic methods is advantageous due to their high selectivity and mild reaction conditions.

On the other hand, in the food industry, the bitter taste of the fresh grapefruit juice has to be adjusted before commercialization to meet the acceptability of the consumers. A variety of physicochemical methods including chemical treatments and adsorption techniques and enzymatic methods have been used to reduce bitterness of these juices.[5] While physicochemical methods can result in a loss of organoleptic properties of the citrus juices, the enzymatic methods have showed high potential for industry. Particularly, naringinase, able to hydrolyze the bitter component of the grapefruit juice, naringin, into non-bitter compounds such as naringenin, glucose and ramnose has been widely applied to sweeten grapefruit juice.[6]

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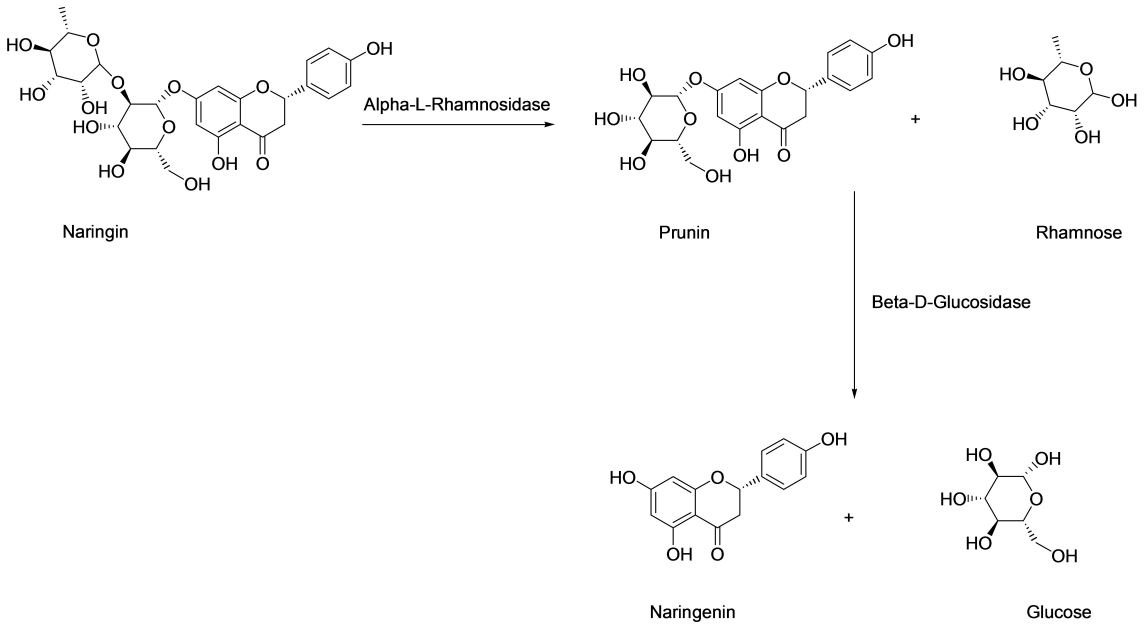

Scheme 1. Pathways in the hydrolysis of naringin.

For industrial biocatalysis, immobilized enzymes on supports can have several advantages respect to the free enzyme such as high thermal or chemical stability, can increase activity, selectivity or specificity, and allows easy separation from the reaction media, and the possibility for reuse. Additionally, immobilization can decrease inhibition problems, resistance to chemicals or increase enzyme purity.[7,8]

Particularly, naringinase has been supported on a variety of materials such as tannin-aminohexylcellulose,[9] chitin,[10] cellulose acetate films,[11] glutaraldehyde coated wood chips,[12] structured mesoporous silicates such as MCM-41,[13] and SBA-15,[14] over sol-gel of polyvinyl alcohol (PVA),[15,16] electrospun cellulose acetate nanofibers,[17] graphene,[18] and graphene oxide[19] while the enzyme has also been entrapped with sodium or potassium alginate.[20–22]

Compared with conventional supports, nanostructured materials possess interesting properties such as their high specific surface area that is beneficial for improving the immobilization efficiency.[23,24] While structured mesoporous molecular sieves such as MCM-41 or SBA-15 with large pore diameter, have potential as supports for enzyme immobilization, they can have limitations associated to its limited stability in aqueous media, and the large steric volume of many enzymes, which can block the channels.

Two-dimensional (2D) zeolites such as ITQ-2 are crystalline materials prepared by delamination of a layered MWW zeolite precursor or by a dual templating one step synthesis,[25–28] resulting in thin zeolite sheets (2.5 nm thick) with a hexagonal array of cups with an aperture of 0.7 nm. (Scheme S1) As consequence, this material presents high external surface area (~600 $m^2 g^{-1}$) containing regularly distributed silanol groups. Moreover, this material is highly stable in aqueous media that, together with their 2D structure, offers the possibility to act as excellent stable enzyme support for industrial biotransformations.[29] In this work, we have immobilized a commercial naringinase (denoted as crude) (from *Penicillium decumbens)* and a purified naringinase with high α-L-rhamnosidase activity (denoted as pure) over pure silica ITQ-2 zeolite. From the hydrolysis of naringin, the crude naringinase allow obtaining selectively the flavonoid naringenin, while the purified naringinase allow obtaining selectively the monoglycoside flavonoid prunin. To immobilize the enzyme, the support has been previously modified by first generating amino groups that are reacted subsequently with glutaraldehyde. Glutaraldehyde is a reagent with high versatility that has been widely used for enzyme immobilization.[30] The aldehyde groups of the modified support (labelled as GITQ-2), can react with nucleophilic groups of the enzyme forming covalent bonds. Thus, a stable enzyme layer with excellent accessibility to reactants is generated, while also limiting the leaching of the enzyme in the reaction media. The enzymatic properties of both immobilized enzymes (crude and pure) have been systematically studied and compared with those of the free enzymes using p-nitrophenyl-alpha-L-rhamnopyranoside (Rha-pNP) as specific substrate model (Scheme S2). The ITQ-2 supported enzymes (crude and pure) have been used in the selective production of naringenin and prunin (highly valuable flavonoids),[3] by hydrolysis of the glycoside naringin that can be extracted from citrus wastes. Finally, their application to grapefruit juice debittering in a continuous fixed bed reactor is presented. We will show how the enzyme supported on the lamellar crystalline silicates compares with the supported on nanocrystalline Beta 3D zeolite, mesoporous MCM-41 material and amorphous silica.

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Characterization of supports and immobilization of naringinase on ITQ-2

For enzyme immobilization, the surface of the pure silica ITQ-2 zeolite was previously functionalized with amino groups by treating the zeolite with 3-(aminopropyl) triethoxysilane (material labelled as NITQ-2). Subsequently, this material was treated with glutaraldehyde yielding a modified ITQ-2 bearing aldehyde groups on the surface (GITQ-2) able to react with amino groups of the enzyme.[25,30]

The ITQ-2, NITQ-2 and GITQ-2 were characterized by FTIR and by elemental analysis (Table S1). In Figure S1(A)(a) are observed the stretching vibration bands corresponding to Si–OH groups of ITQ-2 pure silica at 3741 cm^{-1}. After chemical modification with 3-(aminopropyl)triethoxysilane, the Si–OH band strongly decreases while the stretching vibration bands of amino groups at 3425,1660 cm^{-1} appear, along with the stretching vibration bands of $-CH_2-$ groups (2977 cm^{-1}, 2931 cm^{-1} and 2865 cm^{-1}) associated to the alkyl chain of the 3-(aminopropyl)triethoxysilane (Figure S1(B)(b)). The presence of nitrogen in this sample (NITQ-2) was also confirmed by elemental analysis (Table S1). After glutaraldehyde treatment it is possible to observe the increase in intensity of the vibration bands corresponding to $-CH_2$ groups (see Figure S1(B)(c)) while the appearance of vibration bands at 2842 cm^{-1} and 1725 cm^{-1} associated to C–H and C=O respectively, confirms the presence of aldehyde groups on the surface of GITQ-2, through which the enzyme could be covalently immobilized. FESEM image of GITQ-2 (Figure S2) shows the characteristic sheets of the material able to carry enzymes with huge sizes.

The incubation of 100 mg of this material with naringinase (3 mg) in phosphate buffer at pH 7 afforded the enzymatic derivative Ngsa(3)@GITQ-2. To determine the degree of immobilization with time, samples of the supernatant were taken at different times and the amount of free enzyme was determined by the bicinchoninic acid protein test.[31] Finally, the supernatant was removed from the solid and it was washed with buffer solution until not protein was observed in solution. The results showed that after 24 h of incubation the degree of enzyme immobilization on the GITQ-2 material was $>99\%$ (see Figure S3 and S4), being the activity recovery for the crude and pure naringinase of 91 % and 90 % respectively.

For comparison purposes, the crude naringinase was also immobilized over three different materials, the pure silica mesoporous molecular sieve MCM-41, a pure silica nanocrystalline Beta zeolite, and amorphous silica. Characterization of the modified supports by FTIR spectroscopy (not showed), shows that as for ITQ-2, the characteristics bands after each support modification. In Table S1 the chemical analysis are included. The amount of immobilized enzyme on MCM-41, nanocrystalline Beta zeolite and SiO_2 was considerably lower than that the observed in the case of GITQ-2. In Table S2 can be observed that the order of immobilization is GITQ-2 > GBeta > GMCM-41 $\approx SiO_2$. These results can be explained considering the different structure of the supports that controls the exposed surface to the enzyme during immobilization (see Table S3). Indeed, as can be seen, in the case of the microporous Beta zeolite after glutaraldehyde modification, the BET surface area strongly decreases, while the internal surface area is significantly reduced from 406 to 110 m^2/g indicating micropore blockage of the support after zeolite modification, however, the external surface is practically maintained. In the case of the mesoporous material (MCM-41), which is characterized by a high internal mesopore surface area (1198 m^2/g), the mesopore area is drastically reduced to 49 m^2/g after glutaraldehyde modification due to mesopore blocking during the functionalization of the material. Then, this internal mesoporous surface barely contribute to enzyme immobilization. In the case of amorphous silica, also characterized by a high internal mesopore surface area (476 m^2/g), similar results are observed. Nevertheless, in the case of ITQ-2 zeolite, after glutaraldehyde modification, and due to its lamellar structure the external surface area is maintained quite high (236 m^2/g), leading to a high exposed functionalized surface were the enzyme can be grafted. This should be reflected on the catalyst activity that should be directly proportional to the amount of the enzyme present on the surface of the solid. In this way, in Table S2 the catalytic activity (conversion of naringin achieved in 30 min reaction time) of the different naringinase derivatives is given. As can be seen there, the catalytic activity is clearly related with the amount of enzyme immobilized, i.e. Ngsa(3)@GITQ-2 > Ngsa (3)@GBeta > Ngsa(3)@GMCM-41 $\approx$ Ngsa(3)@$GSiO_2$.

Immobilization performance on GITQ-2

In order to optimize the amount of naringinase that can be supported on GITQ-2, increasing amounts of crude naringinase (3, 6, and 9 mg) were incubated on 100 mg of GITQ-2 following the immobilization procedure described in the experimental section. After 24 h of incubation, the amount of enzyme immobilized was determined by the bicinchoninic acid protein test. In Table S4 the percentage of enzyme immobilized as well as the activity recovery for each sample are presented. As can be observed, the activity recovery is directly related with the amount of enzyme immobilized, showing the maximum percentage of immobilization for the sample Ngsa(3)GITQ-2. Therefore, all the subsequent studies were performed with this sample.

Effect of the pH on enzyme activity

When working with citric juices the pH on of the reactant solution can have an strong impact on activity and stability of the enzyme supported catalyst. Because of that, the effect of the pH on the enzymatic activity of the immobilized naringinase (crude and pure) was determined for the hydrolysis of Rha-pNP in a pH range from 3.0 to 8.0. As can be observed in Figure 1, the optimum pH for the free and immobilized enzyme was the same (4.5). Then, the fact that the supported naringinase on ITQ-2 is able to work with high activity at a pH level similar to

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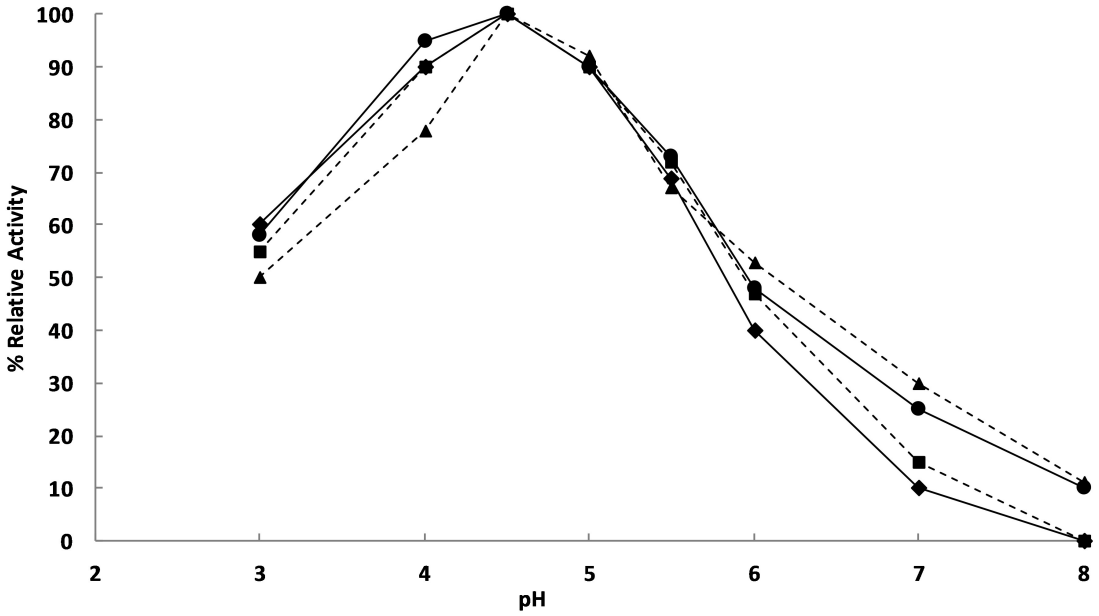

Figure 1. Influence of the pH on the activity of free and immobilized naringinase, Ngsa(3)@GITQ-2(Crude) (●), Free(Crude) (♦), Ngsa(3)@GITQ-2(Pure) (▲) and Free(Pure) (■).

the fruit juices (below 5) is very interesting for large scale applications during fruit juices debittering processes.

Influence of the temperature on the reaction rate

The optimum temperature of operation of an enzyme is determined by the balance between the effect of the temperature on the rate of the enzyme reaction and the rate of the enzyme degradation.[32] Therefore, determination of the optimum temperature is of paramount importance for practical applications. Thus, the influence of the temperature on the α-L-rhamnosidase activity for the free and immobilized enzymes (crude and pure) on GITQ-2 was determined and the results are presented in Figure 2. As can be observed both immobilized enzymes on the GITQ-2 present similar activity with temperature, with the optimum between 55–70 °C. Interestingly the activity of the immobilized enzymes was less affected by the temperature than the free enzyme indicating that the immobilization stabilizes the structure of the enzyme against

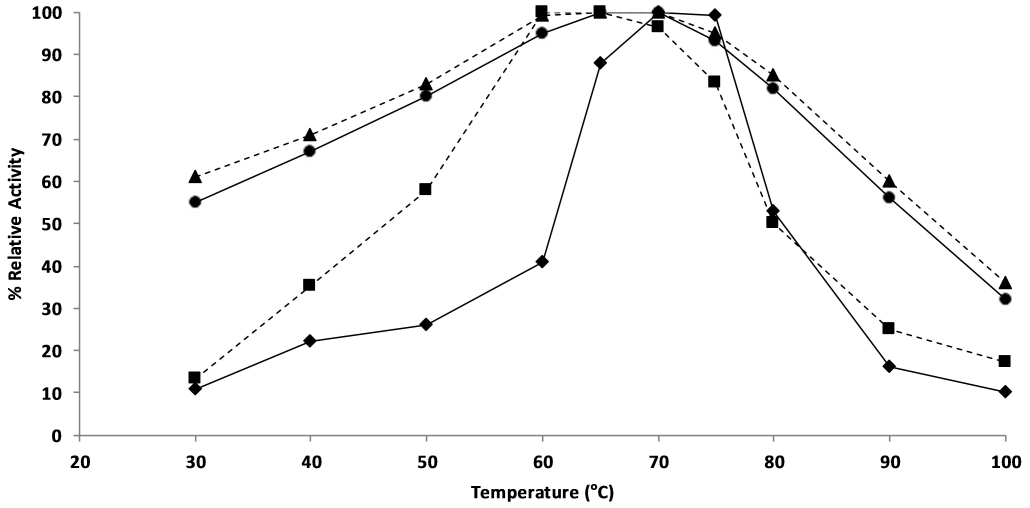

Figure 2. Influence of the temperature on the activity of free and immobilized naringinase. Ngsa(3)@GITQ-2(Crude) (●), Free(Crude) (♦), Ngsa(3)@GITQ-2(Pure) (▲) and Free(Pure) (■).

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denaturation.[33] These results agree with previously reported results[14,34] and with the specific results of thermal stability of immobilized naringinase presented later. An additional experiment performed at 100 °C, using the support (GITQ-2) without enzyme showed that the support has not catalytic activity.

Determination of Michaelis Menten constant (K_M)

To study the effect of substrate concentration on naringinase activity (crude and pure) in free and immobilized (Ngsa(3)@GITQ-2) forms, the reaction rates (under the conditions given in experimental section) were determined at substrate concentrations of Rha-pNP ranging from 0.006–0.312 Mm, while keeping the reaction time constant (10 min). The Michaelis constants (K_M) were calculated by the Lineweaver-Burk method, and the K_M constants for the free enzymes (crude and pure) were very similar (1.87 and 1.80 mM respectively), in good agreement with the results previously reported using Rha-pNP as substrate.[19,35] However, a strong decrease in the K_M values was encountered for the immobilized naringinases, being 0.32 and 0.16 mM for the immobilized crude and pure naringinase respectively. (See Figures S5 and S6). The lower K_M exhibited by the immobilized naringinases compared with free enzymes indicates a higher enzymatic affinity for the substrate which can be ascribed to a higher exposition of the active sites of the enzyme as a consequence of their covalent immobilization on the GITQ-2. The decrease of the K_M after naringinase immobilization was consistent with previous results reported on immobilized naringinase.[11–13]

In Table S5 the K_M reported values of free and immobilized naringinase over different supports are summarized. For comparison purposes we have calculated the CK_M ratios (which is the K_M of the free enzyme divided by the K_M of the immobilized naringinase) for the different biocatalysts. As observed, the CK_M values found for the naringinase supported on ITQ-2 are considerably higher than the previously reported, showing the increase of substrate affinity of the enzyme after their immobilization on GITQ-2.

Thermal stability of free and immobilized enzymes

To determine the thermal stability of free and immobilized naringinase, the biocatalysts were subjected separately to temperatures between 30 and 100 °C for 1 hour. After that, the substrate Rha-pNP was added and the residual catalytic activity was determined under the conditions described in the experimental section. As can be observed in Figure 3 the activity of free and immobilized enzymes remains constant until 70 °C. Nevertheless, at higher temperatures the activity of enzymes immobilized on GITQ-2 are considerably superior to those of free enzymes. For instance, when subjected to 85 °C, the immobilized naringinases (crude and pure) retained 80–90 % of their original enzymatic activity, while the free enzymes only retained 40 % of its catalytic activity. These results indicate that the immobilization caused a marked increase in the temperature stability of the immobilized enzyme. It appears that the GITQ-2 immobilized naringinase is better protected from thermal degradation in comparison to the free enzyme. The higher thermal stability of the immobilized enzyme will be reflected in operational stability, which is an important issue for industrial debittering processes. We will show in the next

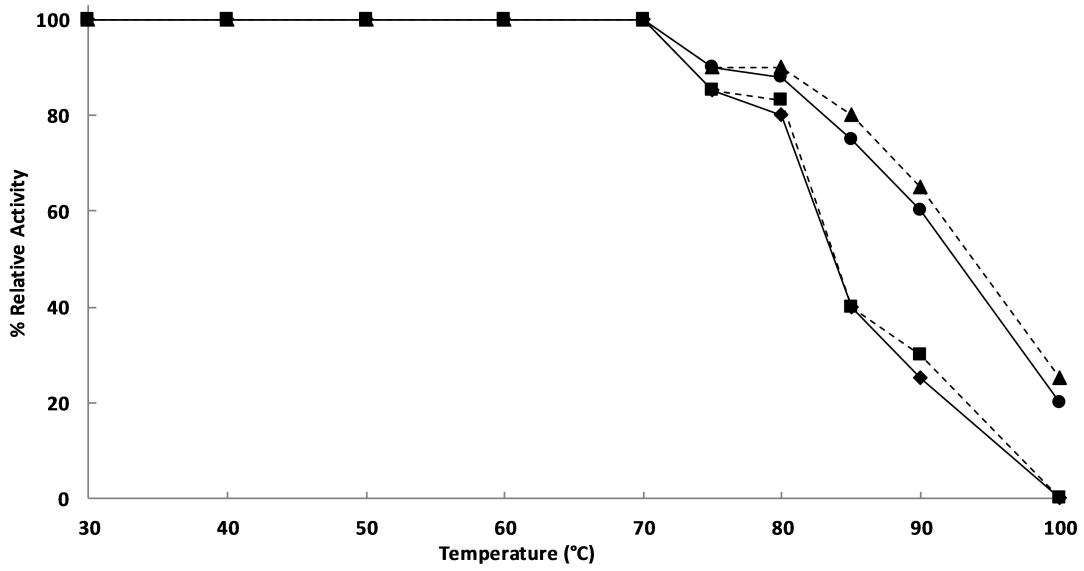

Figure 3. Thermal stability of the free and immobilized naringinase. Ngsa(3)@GITQ-2(Crude) (●), Free(Crude) (♦), Ngsa(3)@GITQ-2(Pure) (▲) and Free(Pure) (■).

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section how the highly stable immobilized naringinase on GITQ-2 allows to perform several reuses without loss of activity.

Catalytic activity and reuse of immobilized enzymes using naringin as substrate

In biological reactions one important advantage of using immobilized enzymes is not only that biocatalysts can be easily separated from the product, but they also enable high operational stability allowing the implementation of continuous reaction processes. These advantages have a significant impact on the cost of the process under practical applications.

Then, to test the stability of the Ngsa(3 mg)@GITQ-2 (crude and pure), we used naringin as substrate. After the first assay, the biocatalyst was separated from the product by centrifugation, repeatedly washed and then submitted to a subsequent cycle. In Figures 4 and 5 the results of activity and selectivity obtained in each reuse for the pure and crude immobilized naringinase are presented. As can be seen there, the conversion of naringin as well as the selectivity to prunin (Figure 4) or naringenin (Figure 5) is practically maintained through 10 consecutive reaction cycles, which indicates the high stability of the biocatalysts. We have calculated the production capacity of each immobilized enzyme after ten cycles being 28 g of prunin per g of pure enzyme and 10 g of naringenin per g of crude enzyme. Moreover, the catalytic experiments for the pure naringinase were continued and not deactivation was observed after 25 cycles, being the production capacity of 70 g of prunin per g of pure enzyme.

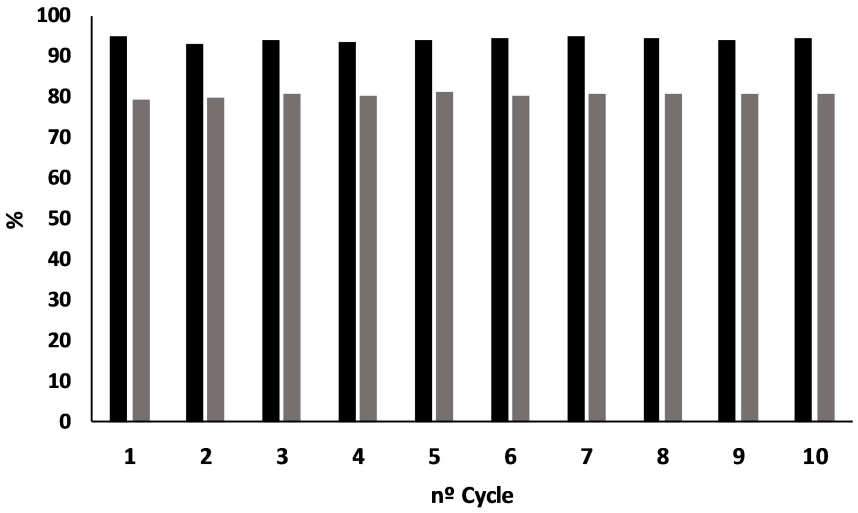

Figure 4. Reuses of Ngsa(3)@GITQ-2(Pure). Reaction conditions: Ngsa(3)@GITQ-2(Pure), 100 mg; naringin, 8.6 mM in 3 mL 50 mM citrate buffer (pH 4.5) at 50 °C for 30 minutes. (■) Conversion, (■) Selectivity to prunin.

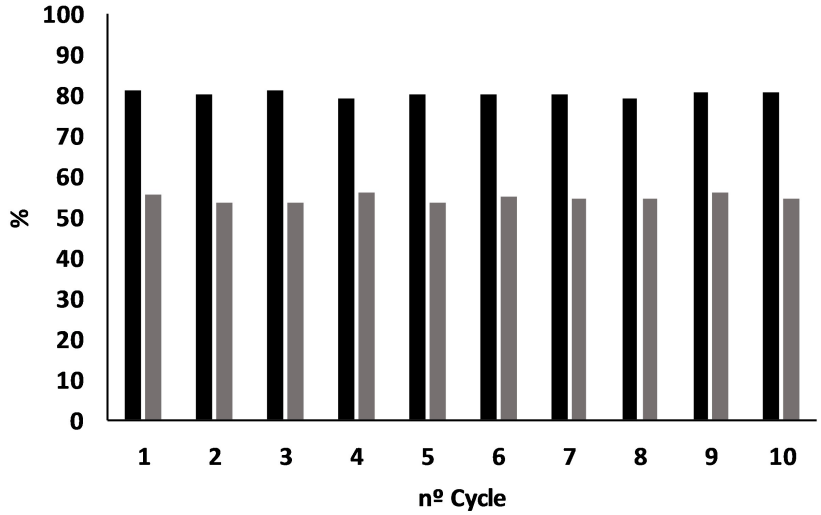

Figure 5. Reuses of Ngsa(3)@GITQ-2(Crude). Reaction conditions: Ngsa(3)@GITQ-2(Crude),100 mg; naringin 8.6 mM, in 3 mL 50 mM citrate buffer (pH 4.5) at 50 °C for 30 minutes. (■) Conversion, (■) Selectivity to naringenin.

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In Table S6 is presented the long term stability and the productivity, i.e. the mg of naringin hydrolyzed per mg of Ngsa (3)@GITQ-2 (crude and pure) (TON) and compared with those previously reported in literature using naringinase immobilized on different supports. As can be observed most of the supported systems reported are not stable or give lower TON values, showing that the productivity of naringinase over our GITQ-2 is superior to those obtained with previously reported systems, and only naringinase supported on graphene oxide (GO) exhibits higher TON. However, the high dispersibility of the GO in water is an important drawback for processing aqueous solutions of naringin or citrus juices in a continuous process in packed bed reactor, due to the possible leaching of the enzymatic derivative. For these processes we will show later that naringinase supported on GITQ-2 is an excellent biocatalyst. These results are of paramount importance for the large-scale production of the high value prunin and naringenin and for debittering citrus juices through a green and competitive method.

Effect of storage time on the stability of immobilized crude naringinase (shelf life)

The activity of the crude naringinase immobilized on GITQ-2 was determined and its remaining catalytic activity was assayed shortly after immobilization (zero day) and with 10 and 20 days of storage at 4 °C (Figure S7). The catalytic activity was maintained after 20 days of storage, showing the high stability of the catalyst over the time.

Grapefruit juice debittering in a continuous process

The excellent results in terms of activity and stability showed by supported naringinase on GITQ-2 prompted us to study their applicability for debittering a grapefruit juice in a continuous process. For that, 200 mg of Ngsa(3)@GITQ-2 crude was placed in a fixed bed tubular stainless-steel reactor that was fed with the grapefruit juice at 50 °C using a perfusion pump. Samples were collected and the amount of sugars released were determined by Miller's method.[36] The influence of contact time on conversion was evaluated changing the flow rate. In Figure S8 the effect of the contact time on the catalytic activity is presented. As can be observed high yields of sugars are already obtained for very reasonable contact times (0.83 h) from a process design point of view. Figure 6 displays the total concentration (g/L) of reducing sugars in the juice as well as the amount of reducing sugars released in the hydrolyzed grapefruit juice using the optimized flow rate (0.25 mL/h). As can be observed (Table S7), the initial concentration of reducing sugars in the grapefruit juice was 61% that increases to 94% after reacting through the fixed bed reactor. The increase observed accounts for a 52% increase in the content of the total reducing sugars. Moreover, no deactivation of the supported enzyme can be observed during 300 h of operation. Considering the total amount of sugars produced and assuming that they came from the hydrolysis of naringin contained in the juice, it is possible to estimate a TON of 40 (g of hydrolyzed naringin per gram of enzyme derivative). These results clearly evidence the high potential of supported naringinase on GITQ-2 to reduce bitterness in the grapefruit juices.[37]

In summary, the crude naringinase from *Penicillium decumbens* and a purified naringinase with high α-rhamnosidase activity have been covalently immobilized on the two-dimensional ITQ-2 zeolite, previous surface modification with gluta-

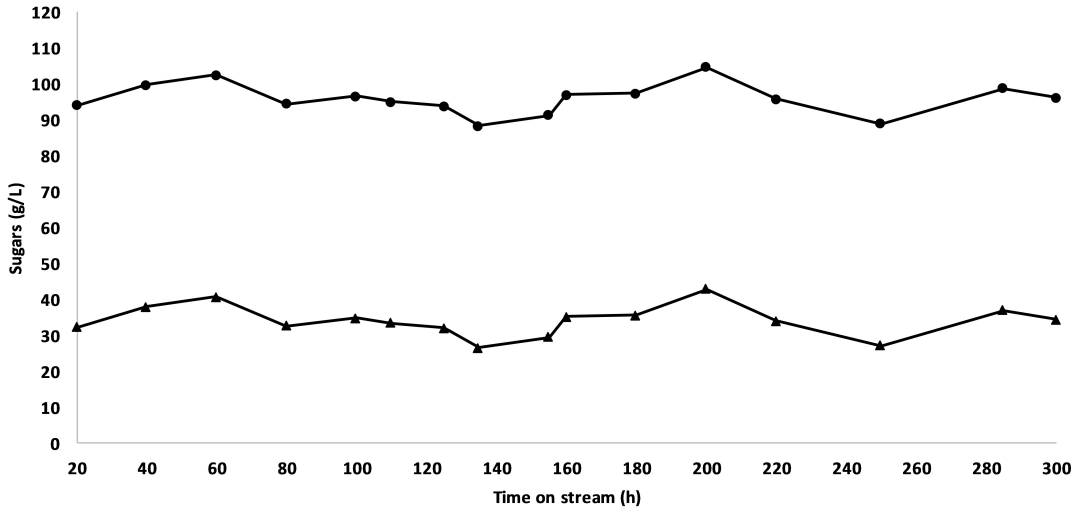

Figure 6. Results of the hydrolysis of grapefruit juice in continuous reactor. Total amount of reducing sugars in the juice (●), amount of reducing sugars released (▲).

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raldehyde. Crude and pure naringinase immobilized on GITQ-2 displayed superior thermal stability and can work in a wider range of reaction temperature, while showing higher affinity for the substrate. The supported naringinases (crude and pure) were applied to the production of two valuable citrus flavonoids prunin and naringenin by selective hydrolysis of naringin. This sustainable and green procedure is clearly advantageous for large-scale production of citrus flavonoids when compared with hydrolysis methods with mineral acids[38] or synthetic methods[39] requiring several steps where low selectivity is achieved. Moreover, the supported naringinase has been used with excellent success in debittering grapefruit juices in a continuous process, maintaining its hydrolysis capacity during, at least, 300 h and showing the high potential of this biocatalyst to reduce bitterness in citrus juices.

Experimental Section

Synthesis of the pure silica MWW (MCM-22) zeolite

The synthesis of pure silica MCM-22 zeolite was performed following the literature.[40] An example of the procedure for the synthesis of MCM-22 is as follows: 0.95 g of NaCl are dissolved in 50.70 g of a solution 0.42 M of N,N,N-trimethyl-1-adamantanamonium hydroxide, previously diluted in 21.33 g of water. Then, 2.62 g of hexamethyleneimine are added to this solution, followed by 4.88 g of silica (Aerosil 200, Degussa) under continuous stirring. This reaction mixture is heated in a Teflon lined stainless steel autoclave at 150 °C rotated at 60 rpm for 5 days. After filtering, the white solid obtained is washed until pH was less than 9, and finally dried at 100 °C.

Preparation of pure silica ITQ-2 zeolite

The synthesis of the pure silica ITQ-2 zeolite was carried out following the literature.[40] Typically, 5 g of the pure silica MCM-22 zeolite were dispersed in 20 g of water. Then, 100 g of an aqueous solution of hexadecyltrimethylammonium hydroxide (25% by weight, 50% exchange Br/OH), and 30 g of an aqueous solution of tetrapropylammonium (40% by weight, 30% exchange Br/OH) were added. The resulting mixture (pH 12.5) was heated to 55 °C and stirred vigorously for 16 h to facilitate swelling between zeolitic sheets. At this point, the suspension was treated in an ultrasonic bath (50 W, 50 Hz) for 1 h to disperse the zeolitic sheets. By adding HCl (6M), the pH was decreased to about 3, to facilitate flocculation of the delaminated solid, which is recovered by centrifugation. Then the solid was washed with distilled water, dried at 60 °C for 12 h, and calcined at 540 °C, first in N_2 for 3 h, and then in air for 6 h.

Synthesis of pure silica nanocrystalline beta zeolite

The synthesis of pure silica nanocrystalline Beta zeolite was carried out according to literature.[41] A gel of molar composition $1SiO_2:0.04Al:0.5TEAOH:6.5H_2O$ was crystallized at 140 °C for 72 h, yielding nanocrystalline zeolite Beta with ca. 50 nm average crystal size and a Si/Al ratio of 21. The zeolite was dealuminated by treatment at 80 °C during 24 h with HNO_3 (60%) in a liquid to solid ratio of 60.[42] The final Si/Al ratio of the seeds was higher than 1000.

Synthesis of pure silica MCM-41

MCM-41 material was prepared from a gel of molar composition: $1SiO_2:0.15C_{16}TMABr:0.26TMAOH:24.3H_2O$, where $C_{16}TMABr$ is hexadecyltrimethylammonium bromide (98%wt, Aldrich), TMAOH is tetramethylammonium hydroxide (25%wt TMAOH in water, Aldrich), SiO_2 (Aerosil, Degussa 200). Typically, 2.8 g of $C_{16}TMABr$ were dissolved in 18 g of MilliQ water at 40 °C until a clear solution was obtained. Then, the solution was cooling down to room temperature and then 4.7 g of TMAOH solution were added, and the resulting mixture was stirred for 5 minutes. Finally, 3 g de SiO_2 are added. The homogeneous mixture was stirred at room temperature for 1 hour. Then, it was heated at 135 °C for 24 hours at the autogenous pressure in a Teflon lined stainless steel autoclave without rotation. The as-prepared MCM-41 sample was recovered by filtration, extensively washed with distilled water and dried at 60 °C overnight. The occluded surfactant was removed by heating the sample at 540 °C under a continuous flow of N_2 for 1 h, followed by 6 h treatment in a flow of air at the same temperature.[43]

Functionalization of the supports with glutaraldehyde

The functionalization of the supports with glutaraldehyde was performed following the literature.[25] The supports (ITQ-2, MCM-41, Beta and SiO_2) (500 mg) were activated at 200 °C under vacuum for 2 hours. After cooling at room temperature, 50 mL of anhydrous toluene and 240 μL of (3-aminopropyl)triethoxysilane were added to the solid and the mixture was refluxed for 24 h under magnetic stirring (500 rpm) at 120 °C . After this time, the solid was filtered under vacuum and washed with toluene and n-hexane, obtaining the material functionalized with amino groups that were denoted as NITQ-2, NMCM-41, NBeta and $NSiO_2$. Then, the material (0.5 g) was placed in contact with 20 mL of a solution of glutaraldehyde (10%) in NaH_2PO_4 buffer (200 mM), at pH 7 and kept under magnetic stirring for 24 h. After this time, the solid was filtered and washed with a buffer solution of NaH_2PO_4 (25 mM) at pH 7. Subsequently, the material was dried at 25 °C, obtaining the glutaraldehyde-functionalized materials labeled as GITQ-2, GMCM-41, GBeta and $GSiO_2$.

Infrared analysis of the supports were performed with a IR Vertex Bruker DTGS (Detector), and a conventional infrared cell Quartz KR55 windows connected to a vacuum dosing system. The samples were pressed into self-supporting pellets and treated under vacuum (10^{-4} to 10^{-5} Pa) at 200 °C for 45 min.

Elemental analysis (Table S1) were performed in a Euro EA3000 Elemental Analyzer (EuroVector), using sulfanilamide as reference.

The specific surface areas of the supports were calculated by the Brunauer-Emmet-Teller (BET) method by means of nitrogen adsorption at −196 °C, using an ASAP 2420 (V2.09J). The results are presented in Table S3.

FESEM Zeiss ultra 55 was used for taking images of the GITQ-2 support. The sample was mounted on carbon tape stacked on aluminum stubs. Image conditions were 1 kV acceleration voltage using the secondary electron detector.

Purification of naringinase

The purification of naringinase was performed following the literature.[19] A solution of naringinase (500 mg in 50 mL of milliQ water) was added to the resin (chromatographic resin DEAE-Sephacel) previously equilibrated with 5 mM NaH_2PO_4 buffer pH 6.8) and maintained under gentle stirring for 1 hour. The adsorption of the enzyme was monitored by protein quantification

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using bicinchoninic acid protein test.[31] The resin was separated from the solution by filtration and the linked protein was desorbed using an increasing gradient of NaCl (0.025 – 1 M). After dialysis, performed to remove salt, the obtained enzyme solutions were lyophilized and analyzed by SDS-Page[19] and then were used in the immobilization assays.

Immobilization of crude and pure naringinase on the glutaraldehyde modified support

The immobilization of the enzyme (crude and pure) on the different supports modified with glutaraldehyde (GITQ-2, GMCM-41, GBeta and $GSiO_2$) was performed as follows: 100 mg of the support were added to 3 mg of enzyme in 3 mL of phosphate buffer solution (100 mM) at pH 7, and left in a closed flask under gentle agitation using a roller bottle for 24 hours. After that, the enzymatic derivative was removed by centrifugation from the supernatant and then the solid was washed with phosphate buffer solution (100 mM), pH 7 and stored at 4 °C. To determine the amount of enzyme immobilized with time, different aliquots of the supernatant were periodically withdrawn and analysed by the bicinchoninic acid protein test. To do this, 2 mL of bicinchoninic acid test solution was added to 0.1 mL aliquot sample and incubated at 37 °C for 30 min. After this time, the optical density of the supernatant was determined spectrophotometrically at λ_{562nm}. Naringinase (crude) was used for calibration curve.[31] These enzymatic derivatives were labelled as Ngsa(3)@GITQ-2, Ngsa(3)@GMCM-41, Ngsa(3)@GBeta and Ngsa(3)@$GSiO_2$.

Immobilization performance of the GITQ-2

The immobilization performance on the GITQ-2 was determined as follows: 100 mg of GITQ-2 were added to 6 and 9 mg of naringinase (crude) dissolved with phosphate buffer solution (100 mM) at pH 7 (1 mg protein/mL), and left in a closed flask under gentle agitation using a roller bottle for 24 hours. After that, the enzymatic derivative was separated by centrifugation from the supernatant and then the solid was washed with phosphate buffer solution (100 mM) pH 7, and stored at 4 °C. To determine the amount of enzyme immobilized, different aliquots of the supernatant were periodically withdrawn and analysed by the bicinchoninic acid protein test. The samples were labelled as Ngsa(6)@GITQ-2, Ngsa(9)@GITQ-2.

Activity recovery calculation

Activity recovery was determined according to literature.[44] Naringin, 8.6 mM in 12 mL of citrate buffer at pH 4.5 (50 mM) was submitted to hydrolysis using 3 mg of free naringinase (pure and crude). The activity was measured and expressed as sugars released ($\mu mol)\cdot min^{-1}$. Then, 3 mg of naringinase (pure and crude) were put in contact with 100 mg of GITQ-2 during 24 hours. After that the solid was recovered by centrifugation and the activity of the total amount of immobilized enzyme was determined under the same conditions as the free enzyme. Activity recovery is expressed as a percentage: (activity of immobilized enzyme/activity of free enzyme) × 100.

Determination of the Michaelis Menten constant (K_M)

The kinetic constants for (crude and pure) enzyme, free and immobilized (Ngsa(3)@GITQ-2), were determined by measuring the reaction rates (under the conditions above mentioned) at substrate concentrations of Rha-pNP ranging from 0.006–0.312 mM and constant reaction time (10 min). Lineweaver-Burk plots were used to determine the K_M.

Effect of the temperature and pH on the free and immobilized enzyme derivatives

Enzyme activity of the crude and pure enzymes in free and immobilized (Ngsa(3)@GITQ-2) forms was determined as describe above, at different temperatures and different pH values (McIlvaine buffer pH 3.0–8.0 range).

Thermal stability of the enzyme.

The stability of the enzyme with the temperature was determined by heating the pure and crude enzyme, in free and immobilized (Ngsa(3)@GITQ-2) forms, at temperatures between 30 and 100 °C in sodium citrate (50 mM) buffer solution pH 4.5 for one hour. Subsequently the substrate Rha-pNP was added and the residual enzyme activity was determined. The initial activity is regarded as 100 %, and residual activity was expressed as a percentage of initial activity.

Catalyst stability

The shelf life of the derivative Ngsa(3)@GITQ-2 was determined and its remaining catalytic activity was assay shortly after immobilization (zero day) and with 10 and 20 days of storage at 4 °C. After each test, the derivative was washed and maintained at 4 °C until further use. The catalytic activity was determined by measuring the sugars released by the Miller method.

Determination of enzyme activity

Naringinase activities were determined in triplicate by incubating a 0.05 mL solution of free or immobilized enzyme (1 mg protein/mL) with 0.05 mL (0.5 mM) of the specific substrate p-nitrophenyl-alpha-L-rhamnopyranoside (Rha-pNP) in 0.7 mL of citrate buffer (50 mM) pH 4.5 at 50 °C for 5 minutes. After this time, the reaction was stopped by adding 0.8 mL of sodium carbonate (1 M). The amount of released p-nitrophenol was monitored colorimetrically at 405 nm using UV-Vis Spectrometer (Varian Cary 50 conc UV-Vis Spectrophotometer). An analytical curve constructed using different solutions of p-nitrophenol provided a value of the molar extinction coefficient of ($\varepsilon_{405nm} = 17.791\ mM^{-1}.cm^{-1}$). One unit of enzyme activity (U) corresponds to the amount of free or immobilized enzyme required to hydrolyze 1 μmol of substrate (Rha-pNP) per minute. Relative activity was expressed taken as 100 % the maximum activity in each case.

Catalytic activity and reuse assay of immobilized enzymes using naringin as substrate

Naringinase performances were determined by incubating the immobilized enzyme Ngsa(3)@GITQ-2 (100 mg) with the substrate naringin (8.6 mM), in 3 mL citrate buffer (50 mM at pH 4.5) at 50 °C for 30 minutes. After reaction, the mixture was centrifuged at 6000 rpm during 10 min, the enzymatic derivative was removed and washed, and used in a subsequent reaction cycle. The analysis of the product was performed by HPLC (Shimadzu LC-20ADXR). The amount of naringin, prunin and naringenin were estimated using Diode Array Detector SPD-M20A, with a Mediterranean SEA18 5 μm 25 × 0.46 column in a gradient mode (Table S8) using water and acetonitrile as mobile phase, with a flow of 0.8 mL/min. The standards for flavonoids were diluted in ethanol and filtered with

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filters Nylon of 0.22 μm. The calibration curves of naringin, prunin and naringenin were done from 0 to 1 mM at λ_{280nm}. The sugars released in the reaction (glucose and rhamnose) were determined using a Refractive Index Detector (RID-20A), with a ICE-COREGEL 87H3 column, in isocratic mode using H_2SO_4 (4 mM) as mobile phase, at a of flow 0.6 mL/min using the column's oven CTO-20 Ac at 75 °C. Fructose was used as internal standard for sugar determination, and the calibration curves with standards sugars samples (glucose and rhamnose) were done at concentrations from 0 to 10 mM. The combination of the two methods allows us to determinate all the different products generated during the hydrolysis of naringin. Reducing sugars were also determined using the methodology described by Miller.[36]

Grapefruit juice debittering in continuous reactor

For the grapefruit debittering test in continuous reactor, four grapefruit variety Mars from Spain Category I, Caliber 4 and weight 0.955 Kg, were squeezed, centrifuged and filtered with Ashless Filters. The juice was homogenized with pH adjusted from 2.5 to 4.5 using NaOH 0.2 M. Then, 200 mg of the Ngsa(3)@GITQ-2 (Crude) were diluted with silicon dioxide ($SiO_2 \geq 0.25$ μm) and packed in a stainless steel reactor of 35 mm long and 1 mm in diameter. The reactor was coupled with an electric heater controller and a peristaltic pump for feeding the reactor. The temperature was fixed at 50 °C for all the experiments, while the contact time was optimized varying the flow rate. Finally, the flow rate was adjusted to 0.25 mL/h. Samples were withdrawn at regular times and the amount of sugars released was determined by the Miller method.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: supported naringinase · ITQ-2 zeolite · naringin · prunin · naringenin · juice citrus debittering

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