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Monitoring Enzymatic Debittering in Grapefruit Juice by High Performance Liquid Chromatography

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ABSTRACT

An HPLC method was developed for monitoring the progress of enzymatic debittering and estimating the efficiency of debitter enzymes by means of simultaneously determining the levels of the bitter flavanone naringin, its hydrolytic intermediate prunin and the tasteless product naringenin in grapefruit juice. The sample preparation was carried out by a polarity modification with anhydrous ethanol, a centrifuge step and a 0.45 μm filtration. Separation was achieved using a reverse phase C-18 column with a mobile phase comprising 30 % acetonitrile. The average recovery rates were above 88 % and the quantitative linearity ranged from 20 - 500 μg/mL for the flavanones analyzed. The conversions of naringin to prunin by α-L-rhamnosidase and prunin to naringenin by β-D-glucosidase in grapefruit juice were monitored, and the efficiency of the individual enzyme was separately evaluated. Applicability of this method in juice processing is also discussed.

Key words: debittering, grapefruit juice, HPLC, α-L-rhamnosidase, β-D-glucosidase and naringinase

INTRODUCTION

Debittering of grapefruit juice is an important process for controlling quality and improving commercial value[1]. Adsorption techniques[2-7] and enzymatic hydrolysis methods[8-11] have been reported to achieve the goal of debittering grapefruit juice. Because of the loss of acidity, sweetness, flavor and turbidity as well as less efficiency in adsorption debittering, enzymatic hydrolysis has showed superior potential in industrial applications[9]. Naringin [4',5,7-trihydroxyflavanone-7-afla-L-rhamnopyranoside-(1,2)-beta-D-glucopyranoside], the principal bitter flavanone glycoside and the primary bitter component in grapefruit juice, can be hydrolyzed by the α-L-rhamnosidase (EC 3.2.1.40) into rhamnose and prunin [4',5,7-trihydroxyflavonone-7-glucopyranoside]. Prunin, with a one third bitterness ratio to naringin, can be further hydrolyzed by the β-D-glucosidase (EC 3.2.1.21) into glucose and tasteless naringenin [4',5,7-trihydroxyflavonone][9]. Since this hydrolytic mechanism can considerably reduce the bitterness in grapefruit juice, many technologies, designed to immobilize or entrap enzymes in reactors, have been developed to achieve the enzymatic debittering of grapefruit juice[9-11].

Naringinase, a crude enzyme obtained from the fungus *Penicillium sp*. or *Aspergillus sp*., provides the activities of α-L-rhamnosidase and β-D-glucosidase and has been widely used in hydrolyzing naringin into naringenin[9]. However, the high cost and limited availability of naringinase has restricted its industrial applicability in the past. Recently, the gene of α-L-rhamnosidase has been cloned and expressed with marked activity in *Escherichia coli*[12]. The recombinant α-L-rhamnosidase provides an economical and an easily available source of debittering enzymes and reveals a practical revolution in industrial debittering of grapefruit juice. Since there are two enzymes involved in debittering, it is important to monitor the hydrolytic progress through determining the activities of both α-L-rhamnosidase and β-D-glucosidase. Thus, developing an analytical method for simultaneously determining the conversions of naringin to prunin and prunin to naringenin is necessary.

Colorimetric and chromatographic analyses as well as immunoassays are used to analyze the contents of naringin or prunin in the fruit and juice of grapefruit. The colorimetric assays mostly diversified from the Davis test[13-15] are not specific in differentiating between naringin, its tasteless isomer narirutin and its hydrolytic products. Although immunoassays provide great sensitivity (2 ng/mL) in naringin determination[16], it is impractical for routine analysis because of its cross reactivity to other flavonoids in the juice, inability to do simultaneous analysis with other flavonoids, is time consuming and not economical. On the other hand, analysis accomplished by high performance liquid chromatography (HPLC) is frequently used in determining the levels of flavonoids or flavanone in citrus juice[13, 17-19]. However, most reports applying HPLC were only to quantify the naringin content in grapefruit juice. There are no studies available that simultaneously determine naringin and its hydrolytic products, prunin and naringenin, nor any that focus on monitoring the efficiency of both α-L-rhamnosidase and β-D-glucosidase during the debittering process, especially for grapefruit juice samples.

The objective of this study was to find a method for monitoring the progress of enzymatic hydrolysis of naringin in grapefruit juice. This method used HPLC to simultaneously determine the contents of the flavanone glycosides, naringin and prunin, and their aglycone naringenin. The effi-

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ciency of α-L-rhamnosidase and β-D-glucosidase can be further calculated. The results are helpful in developing debittering processes for the grapefruit juice industry.

MATERIALS AND METHODS

I. *Materials*

Grapefruit juice samples were obtained from fresh grapefruits (*Citrus paradisi*, Macf) purchased from local commercial outlets in Taipei. The fruits chosen were at the mature stage with firm texture, uniform color and no sign of spoilage. The juice samples were extracted by squeezing and filtrating to remove the seeds and pomaces.

Flavanone standards naringin and naringenin as well as enzymes naringinase (Product NO. N1385, 300 IU/g) from *Penicillium decumbens*, α-L-rhamnosidase (Product NO. H8510, 10 IU/g) from *Penicillium sp*. and β-D-glucosidase (Product NO. G4511, 30 IU/mg) from Almonds were obtained from Sigma (St. Louis, Mo). Prunin was supplied by Indofine (Somerville, NJ). All solvents were obtained from E. Merck (Schuchardt, Germany).

Stock solutions of naringin, prunin and naringenin were prepared at a concentration of 1 mg/mL by dissolving in 50% aqueous ethanol. The working solutions and the simulative samples were prepared at desired concentrations by diluting the stock solutions with 50% aqueous ethanol and 0.1 M sodium acetate buffer (pH 3.7), respectively.

II. *Methods*

(I) *Sample Preparation*

One milliliter of the stimulatory, juice or hydrolyzed sample was pipetted and vigorously mixed with 1 mL anhydrous ethanol. The mixture was then centrifuged at 10000 rpm ($1.69 \times 10^4 \times g$) for 10 min. The collected supernatant was filtered through a 0.45 μm nylon membrane and further analyzed by HPLC.

(II) *Enzymatic Hydrolysis*

Enzyme solutions were prepared by dissolving naringinase, α-L-rhamnosidase or β-D-glucosidase in 0.1 M sodium acetate buffer (pH 3.7) at a concentration of 10 mg/mL (3, 0.1 and 300 IU, respectively) . Ten microliters of an enzyme solution (10 mg/mL) were added to one milliliter of the stimulatory or juice sample. The reaction was performed at 37°C for 5 to 60 min according to the requirement of each experiment. After reaction, the hydrolyzed sample was immediately prepared and the contents of naringin, prunin and naringenin were determined.

The enzyme efficiency was taken as the amount of substrate liberated (μmol) / protein (mg) / reaction time (min).

(III) *HPLC Analysis*

A Jasco LC-800 HPLC system equipped with a Model 880-PU pump, Model 870-UV detector and Model 851-AS autosampler was used for analysis. Separations were performed on a reverse phase RP-18e (5 μm) column (Merck KGaA, Darmstadt, Germany). The mobile phase was acetonitrile-water (30:70, v/v) programmed at a flow rate of 0.8 mL/min. The injection volume was 20 μL and the column was at ambient temperature. Naringin, prunin and naringenin in samples were detected at 280 nm and identified by comparison of their retention times with those of standards.

(IV) *Calculation and Statistical Analysis*

Standard curves were made by means of injecting the working solutions at various concentrations ranging from 1 - 1000 μg/mL. The levels of naringin, prunin and naringenin were determined using response factors obtained with the single-point external calibration method. For the recovery calculation, 50 - 500 μg/mL of standards were spiked into the juice samples and compared with their concentration obtained by HPLC analysis. The limit of detection was determined as a concentration giving the response at a signal-to-noise ratio above 5 ($s/n > 5$). The limit of quantification was determined as a concentration sustaining a correlation coefficient of a standard curve above 0.995.

Three analyses of each sample were made and each experiment was carried out in triplicate (n = 3). The results are expressed as means ± SE. Statistical comparisons were made by one-way analysis of variance (ANOVA) followed by a Dunnett multiple comparisons test. Differences were considered significant when P-values were less than 0.05.

RESULTS AND DISCUSSION

I. *Sample Preparation and Analytical Characteristics*

To evaluate the efficiency of the two enzymes involved in grapefruit juice debittering, a simultaneous analysis for the bitterness substrate naringin, the intermediate prunin and the tasteless product naringenin is desired. Unfortunately, the polarity of the glycosides naringin and prunin is much higher than that of their aglycone naringenin, which makes the analysis difficult. Since the aglycone naringenin is almost insoluble in water, an additional procedure of adding ethanol solution to the juice in the sample preparation was made to modify the polarity of the solution and increase the recovery of naringenin. The influence of ethanol content in the prepared sample on the recoveries of flavanones is shown in Figure 1. The decrease of polarity in the prepared sample solution increased the recovery of naringenin but reduced that of naringin and prunin. According to this result, 50 % ethanol obtained by adding equal volume of anhydrous ethanol to juice samples was found to give reasonable recoveries (above 90 %) for all analyzed flavanones and was used in further examinations.

Chromatographic parameters, which are able to separate both hydrophilic and hydrophobic compounds, were consid-

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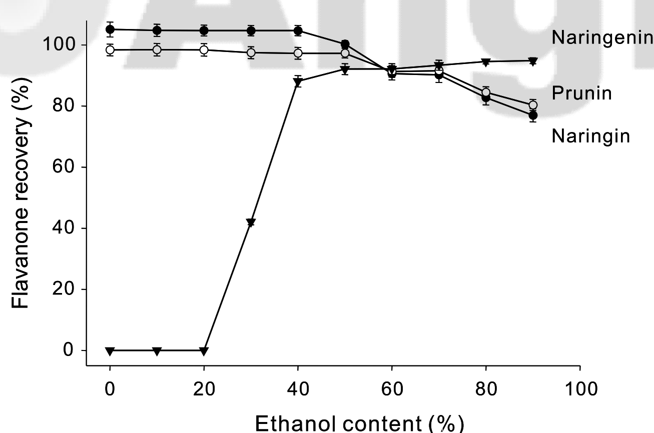

Figure 1. Effect of polarity modification by addition of ethanol on the recovery of flavanones naringin (●), prunin (❍) and naringenin (▼) in the simulative samples containing 200 μg/mL flavanone. The polarity modification was carried out by adding anhydrous ethanol to the simulative sample at various volume ratios in sample preparation. Recovery (%) was calculated as the detected level of the flavanone divided by the original concentration of that in the simulative sample.

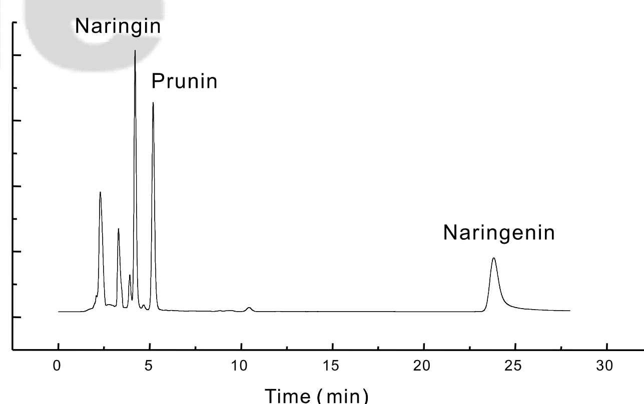

Figure 2. Chromatogram of naringin, prunin and naringenin in grapefruit juice sample. The flavanones were spiked into the juice sample at the same concentration of 500 μg/mL. The retention times of naringin, prunin and naringenin were 4.82, 5.22 and 23.04 min, respectively.

ered for the simultaneous analysis. The separation was carried out by a reverse phase C_{18} column and a constant mobile phase of acetonitrile-water system. The chromatogram (Figure 2) showed the baseline separation of naringin, prunin and naringenin with a retention time of 4.82, 5.22 and 23.04 min, respectively. The analytical characteristics are summarized in Table 1. The linear range of naringin, prunin and naringenin covered the levels of these flavanones in grapefruit juice. In addition, the high linearity of response and the coefficient of linear regression (r^2) with confidence better than 0.998 for each flavanone was observed. The recoveries of naringin and prunin exceeded 95 % in most levels spiked to the sample with the coefficient of variety under 2%. However, the recovery of naringenin was decreased with increased naringenin levels. Moreover, the detection limit, calculated as the minimum sample size to produce a signal to noise ratio of 5, was 1, 5 and 0.5 μg/mL for naringin, prunin and naringenin, respectively, in grapefruit juice. An analysis could be completed in 25 min. These analytical characteristics indicated that it was an ideal method for the simultaneous determination of naringin, prunin and naringenin in juice samples.

Most reports have discussed the analysis of naringin in grapefruit juice only. As additional prunin and naringenin with opposite polarity were included in this study, many parameters of sample preparation and HPLC analysis were optimized for simultaneous analysis. Simple procedures in sample preparation consisted of a polarity modification in solution and a centrifugal step followed by a 0.45 μm filtration provided adequate recovery (Table 1.) and good background cleanup (Figure 2). The step of modifying the solution polarity came with an additional advantage as it was able to stop the enzyme reaction (data not shown). In such a 50% ethanol-juice solution, proteins in juice might be denatured and the debittering enzymes should be inactivated. Some soluble pectin substances and proteins, which might disrupt the analysis, could be precipitated by adding ethanol and removed by following with centrifugation and filtration. Moreover, the analytical characteristics showed that this method provides good sensitivity, great quantitative properties and short analyzing time. Thus, this simultaneous analysis could be suitable for supervising the conversions of bitter flavanones in grapefruit juice.

Table 1. Chromatographic characteristics of the analysis of naringin, purnin and naringenin by HPLC

	Naringin	Prunin	Naringenin
Linear range (μg/mL)	20–500	10–1000	5–500
Linearity (r^2)[a]	0.9985	0.9998	0.9996
LOD (μg/mL)[b]	1	5	0.5
Recovery of spiked sample (%)[c]			
Spiked levels (μg/mL)			
50	99.6 ± 1.9	98.2 ± 1.1	93.4 ± 2.1
100	100.3 ± 1.4	97.3 ± 1.4	92.1 ± 1.9
250	104.1 ± 1.7	95.7 ± 1.4	88.5 ± 1.7
500	103.2 ± 1.9	96.2 ± 1.8	82.2 ± 2.7

[a] The response coefficients (r^2) of linear regression. A five-point regression of the standard curve was made for each compound.
[b] Limit of detection (LOD) of additive added in grapefruit juice. LOD was calculated as the minimum sample size that will produce a signal five times that of the short-term noise level.
[c] Averaged recovery (%) ± CV%. The coefficient of variation (%CV) was obtained from triplicate injections.

II. *Monitoring the Progress of Enzymatic Debittering in the Simulative Sample*

To evaluate the analytical method and to overlook the flavanone conversion, α-L-rhamnosidase and β-D-glucosidase were sequentially added to the simulative samples and the levels of naringin, prunin and naringenin were determined (Figure 3A). At the fifth min, α-L-rhamnosidase (0.1 mg/mL) was added to a simulative sample containing 500 μg/mL of naringin and the conversion of naringin to prunin was observed. The first hydrolysis could be completed in about 30 min. Similarly, β-D-glucosidase (0.1 mg/mL) was then added at the 25th min and the conversion of prunin to

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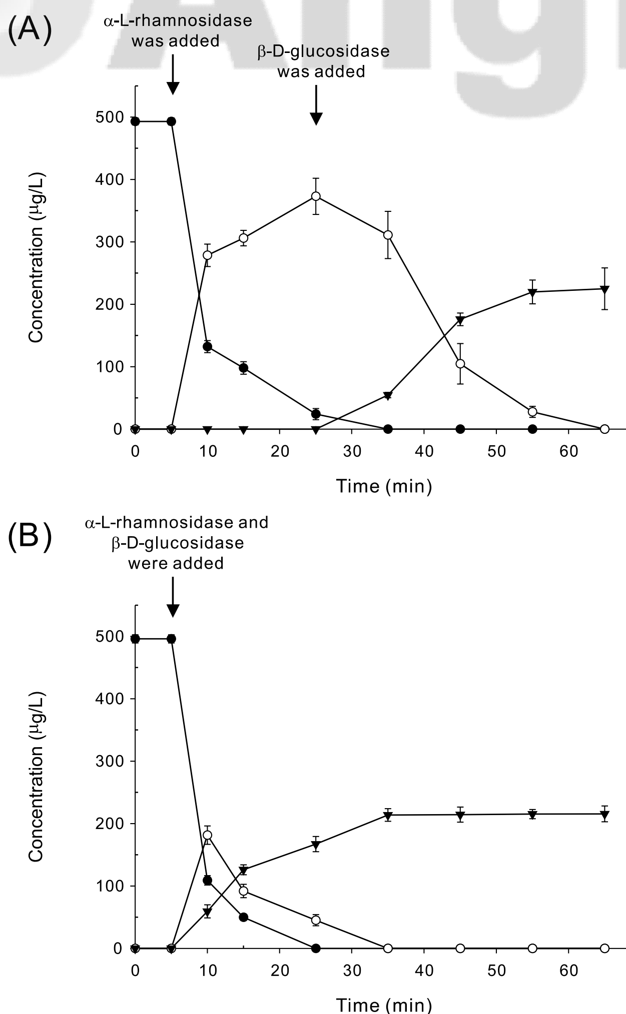

Figure 3. The hydrolytic conversions of naringin (●) to prunin (❍) and prunin to naringenin (▼) by α-L-rhamnosidase (0.1 mg/mL) and β-D-glucosidase e (0.1 mg/mL) in the simulative sample containing 500 μg/mL naringin. (A) α-L-rhamnosidase and β-D-glucosidase were sequentially added to the samples at the 5th min and the 25th min, respectively; (B) α-L-rhamnosidase and β-D-glucosidase were simultaneously added to the samples at the 5th min.

naringenin was observed. It took another 40 min to finish the second hydrolysis. The hydrolytic efficiency of α-L-rhamnosidase and β-D-glucosidase was calculated as 41.2 and 20.7 μmole/mg/min, respectively. However, it was found that naringin could not be hydrolyzed by β-D-glucosidase only (data not shown), which showed that the sequence of this hydrolytic procedure is irreversible. Results clearly demonstrated the rise and fall of naringin, prunin and naringenin during these hydrolysis processes. This method is useful for simultaneously supervising the activity of both α-L-rhamnosidase and β-D-glucosidase.

The cooperative hydrolysis of α-L-rhamnosidase and β-D-glucosidase on naringin in the simulative sample was also investigated (Figure 3B). The hydrolytic process showed that β-D-glucosidase could convert prunin into naringenin immediately after the formation of prunin from naringin by α-L-rhamnosidase. Moreover, the reaction time could be reduced to 30 min, which was much shorter than that in the sequentially hydrolyzed treatments (Figure 3A). There is no significant difference in the efficiency change for both enzymes when sequentially or simultaneously used. Results indicated that these two enzymes could function together without interruption. Although there were only free enzymes used in this experiment, it is believed that the efficiency of these two enzymes could be individually and simultaneously monitored using this analytic method when the enzymes are separately immobilized in linked columns for industrial applications.

III. *Evaluating the Debittering Efficiency in Juice Samples*

The applicability of this analytical method on evaluating the debittering efficiency in juice samples was evaluated. The conversions of naringin to prunin and prunin to naringenin by commercial naringinase (0.1 mg/mL) (Figure 4A) and the enzyme mixture (Figure 4B) in grapefruit juice were successfully monitored by this method. The other juice solutes seemed not to interfere with the quantification of these flavanones by the HPLC analysis. Moreover, lower efficiency of

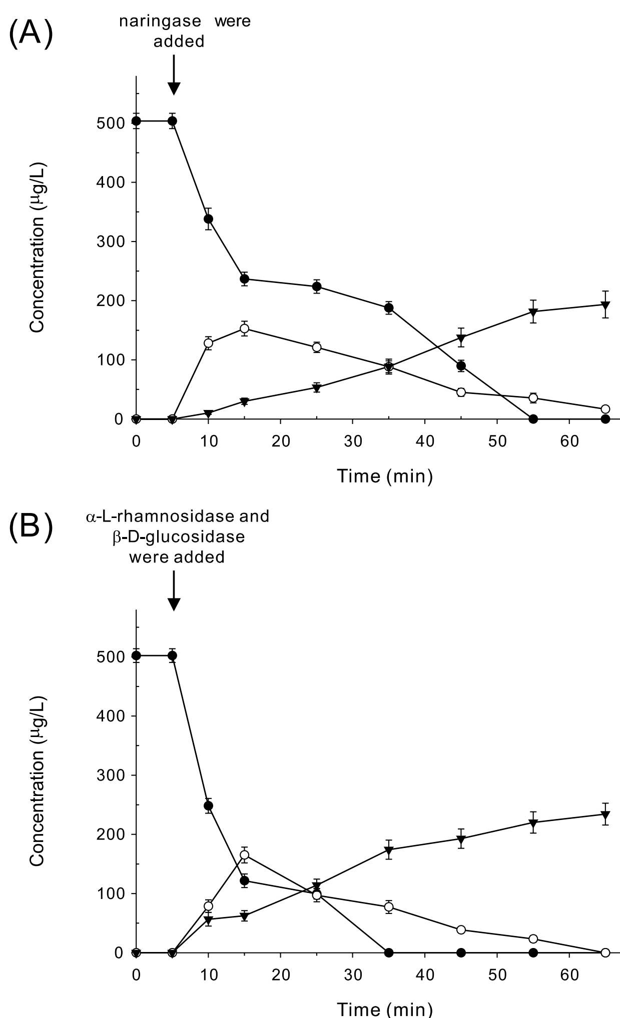

Figure 4. The hydrolytic conversions of naringin (●) to prunin (❍) and prunin to naringenin (▼) by (A) commercial naringinase (0.1 mg/mL) and (B) an enzyme mixture of α-L-rhamnosidase (0.1 mg/mL) and β-D-glucosidase (0.1 mg/mL) in grapefruit juice samples. The naringin level in the juice blank was determined as 503.2 μg/mL.

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α-L-rhamnosidase (17.4 μmol /min/mg) and β-D-glucosidase (11.9 μmol/min/mg) in naringinase were also observed. The reactions for removing naringin took 30 min by the enzyme mixture of α-L-rhamnosidase and β-D-glucosidase (Figure 4B) but took 50 min by naringinase (Figure 4A). The lower efficiency of naringinase may result from a crude enzyme and lack of advanced purification for naringinase.

The debittering efficiency corresponds to the specific activities of the enzymes used. Since there are two different enzymes involved in the hydrolytic reaction, the lifetimes and the retentive activities of α-L-rhamnosidase and β-D-glucosidase should be considered separately. Therefore, purified enzymes or recombinant enzymes are suggested for industrial applications in debittering grapefruit juice. When immobilizing techniques are used for continuously debittering, α-L-rhamnosidase and β-D-glucosidase could be packed in separate reactors, simultaneously monitored by this method and individually replaced while the activity of each enzyme is decayed. On the other hand, the crude naringinase could be immobilized only as the form of an enzyme mixture. If one of the hydrolytic enzymes is inactivated or decayed, the other enzymes should be discarded together. In addition, the variable composition and various activity of naringinase make it unsuitable in the adaptation of juice engineering. Nevertheless, the present method is able to determine the hydrolytic efficiency, measure the immobilizing efficiency, and calculate the half-life of the debitter enzymes in juice samples.

Colorimetric analysis has been reported to determine the activities of naringinase in buffer solutions but not in juice samples. The other flavonoids in grapefruit juice can react with the reagents and give a high background noise, which makes the quantification of naringin and other flavanones unattainable. In contrast, this chromatographic analysis accomplished by HPLC provides a simultaneous determination of the bitterness substrate naringin, the intermediate prunin and the tasteless product naringenin. It is the first reported method that focuses on evaluating the efficiency of both α-L-rhamnosidase and β-D-glucosidase in grapefruit juice.

CONCLUSIONS

A method of sample preparation and HPLC analysis was developed for the simultaneous determination of naringin, prunin and naringenin. The method provided good analytical characteristics and was capable of investigating the decrease of naringin, monitor the conversions of bitter flavanones to tasteless ones, and evaluate the efficiency of the debittering enzymes α-L-rhamnosidase and β-D-glucosidase. It is helpful for studying the enzyme kinetics of debittering enzymes in juice processing.

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利用液相層析法偵測以酵素去除葡萄柚汁苦味的過程

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摘　要

本研究發展一液相層析方法，用以同時偵測酵素去除葡萄柚汁苦味的過程中，柚苦甙（naringin）及其水解產物洋夸甙（prunin）、柚苦素（naringenin）等黃烷酮物質的含量變化，並可估算去苦味酵素的效率。葡萄柚汁樣品經添加無水酒精改變極性後，再經離心及過濾步驟製備，層析分離於逆相碳十八管柱以及含30% 氰甲烷水溶液之動相完成。三種黃烷酮之回收率均大於88% 而定量線性範圍介於20 - 500 μg/mL。此法可觀察葡萄柚汁去苦味過程中，α-L-rhamnosidase將柚苦甙水解為洋夸甙，以及β-D-glucosidase將洋夸甙水解為柚苦素的變化，也可用來分別估算去苦味酵素α-L-rhamnosidase及β-D-glucosidase的水解效率，因此適於日後葡萄柚汁工業化去苦味的應用。

關鍵詞：去苦味，葡萄柚汁，高效能液相層析，鼠李糖甙�THE，葡萄糖甙�THE，柚苦甙�THE