

# Validation of High-Performance Liquid Chromatography for Determination of Bromelain in Pineapple (*Ananas comosus* (L) Merr) Water

Novi Yantih<sup>1,\*</sup>, Alfadella Methananda<sup>1</sup>, Yahdiana Harahap<sup>2</sup>, Wahono Sumaryono<sup>1</sup>, Lestari Rahayu<sup>1</sup>

Novi Yantih<sup>1,\*</sup>, Alfadella Methananda<sup>1</sup>, Yahdiana Harahap<sup>2</sup>, Wahono Sumaryono<sup>1</sup>, Lestari Rahayu<sup>1</sup>

<sup>1</sup>Faculty of Pharmacy, Universitas Pancasila, South Jakarta, Jakarta, INDONESIA.

<sup>2</sup>Faculty of Pharmacy, Universitas Indonesia, Depok, West Java, INDONESIA.

## Correspondence

Novi Yantih

Faculty of Pharmacy, Universitas Pancasila, South Jakarta, Jakarta, INDONESIA.

E-mail: novi\_yantih@yahoo.com

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## ABSTRACT

**Objective:** The aim of the present study was to validate HPLC method for analysis bromelain levels in pineapple water and application that method to determine the storage time of pineapple water. **Methods:** The reversed phase of HPLC method was tested and optimized before it is validated. The composition and the flow rate of the mobile phase is the optimized parameter. The analytical parameters validated were detection limits, linearity, accuracy and precision. Pineapple water was stored for 8 h at 10°C and bromelain was determined using the validated HPLC method. **Result:** The optimum mobile phase composition was methanol-water (70:30) with a flow rate of 1 ml/min. The detector concentration-response was linear with coefficient of determination of 0.993. The accuracy of HPLC method at a recovery of 1 and 2% bromelain was  $106.37 \pm 1.94\%$  and  $98.12 \pm 1.29\%$  ( $n = 5$ ), respectively. The precision, expressed as the coefficients of variation (CV), at 1 and 2% bromelain were 1.83 and 1.32% ( $n = 5$ ), respectively. Bromelain level at zero time was 81.53%. After storage for 8 h at 10°C, bromelain levels in pineapple juice appeared to decrease not statistically significant ( $p > 0.05$ ), with to mean value of  $78.46 \pm 2.88\%$ . **Conclusion:** The HPLC method developed was valid to analyze accurately concentrations of bromelain in pineapple water and it can be used to study the shelf life of pineapple water based on bromelain content. The bromelain content in pineapple juice was not statistically significantly different after 8 h storage at 10°C.

**Key words:** Bromelain, HPLC, Pineapple water, Storage.

## INTRODUCTION

Pineapple fruit (*Ananas comosus* (L) Merr) contains nutrients that are beneficial to health. Nutritional content in pineapple fruits include water, protein, fat, carbohydrates, calcium, phosphorus, iron, vitamin A, vitamin B and vitamin C.<sup>1</sup> In addition, pineapple fruit also contains protease enzymes including ananase, comosain and bromelain. Bromelain levels concern the highest protease enzyme in pineapple. Bromelain can be found in all tissues of pineapple plants, including the stem, fruit and leaves.

Pineapple fruit has pharmacological benefits, because it contains high bromelain concentrations.<sup>2</sup> Bromelain possesses multiple therapeutic activities. *In vitro* and *in vivo* studies demonstrate that bromelain exhibits various fibrinolytic, antiedematous, antithrombotic and anti-inflammatory activities. Bromelain is considerably absorbable in the body without losing its proteolytic activity and without producing any major side effects. Bromelain accounts for many therapeutic benefits like the treatment of angina pectoris, bronchitis, sinusitis, surgical trauma and thrombophlebitis, debridement of wounds and enhanced absorption of drugs, particularly antibiotics. It also relieves osteoarthritis, diarrhea and various cardiovascular disorders. Bromelain

also possesses some anticancerous activities and promotes apoptotic cell death.<sup>3-5</sup> Bromelain proved to be effective at controlling postoperative pain at 48–72 h after surgery, but did not achieve a significant effect in comparison to the control group with regard to oedema or trismus.<sup>6</sup>

Pineapple fruit consumption is one way to get nutrients in pineapple. Pineapple fruit water is consumed directly after the manufacturing process or after being stored for a while after the manufacturing process. Shelf life is the time within which a product remains stable. This can be established from chemical properties during storage. Based on vitamin C level, shelf life studies of pineapple juice have been reported.<sup>7,8</sup> Bromelain can be used as an indicator of the quality of pineapple fruit, because bromelain is the largest component of the protease enzyme in pineapple.<sup>2</sup>

In general, enzymes acting as biocatalysts have an unstable nature in their environment.<sup>9</sup> Enzyme levels and activities can be affected by temperature, pH and physical modification during storage. Bromelain is sensitive to high temperatures, acidity and organic solvents.<sup>10-12</sup> This instability will result in decreased enzymatic activity and limit its pharmacological effects.<sup>2</sup> Consequently, a study is needed to determine the bromelain content during storage of pineapple fruit water. In order to determine the bromelain contents, a validated analytical method is needed.

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Several chromatographic techniques, such as ion exchange chromatography, high-speed counter-current chromatography, affinity chromatography, gel filtration chromatography and capillary electrochromatography have been employed for the purification of bromelain.<sup>3,13</sup> Gradient elution of high performance liquid chromatography (HPLC) has been reported to determine bromelain in crude bromelain<sup>14</sup> and stem bromelain.<sup>5</sup> Bromelain has been quantified by visible spectrophotometry in tablets<sup>15</sup> and by HPLC in gel formulation.<sup>16</sup> Determination of bromelain content in pineapple water by isocratic HPLC method using specific column and UV detection has been reported.<sup>17</sup> Therefore, this study aims to develop and validate a novel isocratic HPLC method with photodiode array detector for the analysis of bromelain contents in pineapple fruit water. The validated method is also used to determine the effect of storage of pineapple fruit water on levels of bromelain.

## MATERIALS AND METHODS

### Materials

Pineapple (*Ananas comosus*), Smooth Cayenne type, was obtained from Subang, West Java, Indonesia. The fruit was determined at the "Herbarium Bogoriense" Botanical Field of the Biological Research Central, Indonesian Institute of Sciences, Cibinong-Bogor, Indonesia. Bromelain (Sigma Aldrich, Missouri, United State of America) was used as Reference standard. Methanol (Merck, Darmstadt, Germany) was pro HPLC grade.

**Equipments:** The main equipment components used in this research were a Knauer Smartline S2600 (Knauer, Berlin, Germany), a juicer (Kuche, Jakarta, Indonesia) and Eppendorf tubes (Eppendorf, Hamburg, Germany).

### Methods

**Preparation of pineapple water:** Pineapple fruit was cut into small pieces and then mixed with a juicer, then put in a glass container and covered with plastic wrap and stored in a refrigerator at 10°C.

**Optimization of mobile phase of HPLC system:** Selection of the mobile phase was carried out to obtain the mobile phase composition which can provide effective and efficient results in HPLC analysis. Chromatograph conditions were supported with C18 (150 mm × 4,6 mm) as stationary phase, methanol-air as mobile phase with flow rate of 0.8, 1.0 and 1.2 mL/min and photodiode array (PDA) as a detector at 230 nm. The system suitability test was done before analysis.

**Calibration curve:** Calibration curves were made to determine the concentration of analytes in the sample. The series solutions of bromelain with concentrations of 1, 2, 4, 6, 8 and 10% were prepared in methanol. A total of 20 µl of solution was injected into the chromatograph at each concentration. The curve is made from the relationship between analyte and concentration.

### Validation of HPLC methods

**Linearity test:** A total of 8 sample concentrations were made to test linearity. Pineapple juice samples as much as 0.1; 0.2; 0.4; 0.6; 0.8; 1.0; 1.2; and 1.4 mL were inserted into each measuring flask, then added with methanol to 5 mL. A total of 20 µl of solution from each concentration was injected into the chromatograph. The correlation coefficient (r) of the curve is made from the relationship between analyte and concentration.

**Accuracy test:** Determination of the accuracy of the method was using the addition method of 1% and 2%. A total of 20 µl of solution was injected into the chromatograph as many as 5 times of each concentration. Accuracy can be determined by calculating of recovery. **precision test:** Determination of the precision of the method was

using the addition method of 1% and 2%. A total of 20 µl of solution was injected into the chromatograph as many as 5 times of each concentration. Precision was calculated as the coefficient variance of each concentration.

**Determination of bromelain in pineapple fruit water:** Pineapple fruit water is stored for several times, namely 0, 1, 2, 4 and 8 h. Bromelain levels were analyzed at each storage time. Sample was filtering using filter paper. Filtrate was pipetted as much as 0.4 mL and put into a 5 mL flask and added with methanol to 5 mL, then homogenized. The solution was filtered with a 0.2 µm membrane filter and sonicated for 15 min before injected to chromatograph.

## RESULT AND DISCUSSION

### Optimization of mobile phase of HPLC system

In the method optimization, a composition of methanol-water ratio (70:30) with a flow rate of 1 mL/min was perform as a mobile phase. The chromatograms can be seen in Figure 1 and Table 1. From Figure 1, the peak of bromelain was appears in a 2-min time retention time. Peak area of the analyte was greater at a flow rate of 1 mL/min than 0.8 and 1.2 mL/min. This showed that the sensitivity of the method increased at a flow rate of 1 mL/min.

This proposed method is more efficient than reported method,<sup>5,16</sup> since the retention time of bromelain appeared at 2 min.

### System suitability test

In the system suitability test, the standard solution of bromelain was at a concentration of 6%. The test results showed the CV value of the area and retention time (n = 5) of 1.07 and 1.49%, respectively. This value indicates a system suitability met the requirements with KV of ≤ 2% (18).

### Linearity test

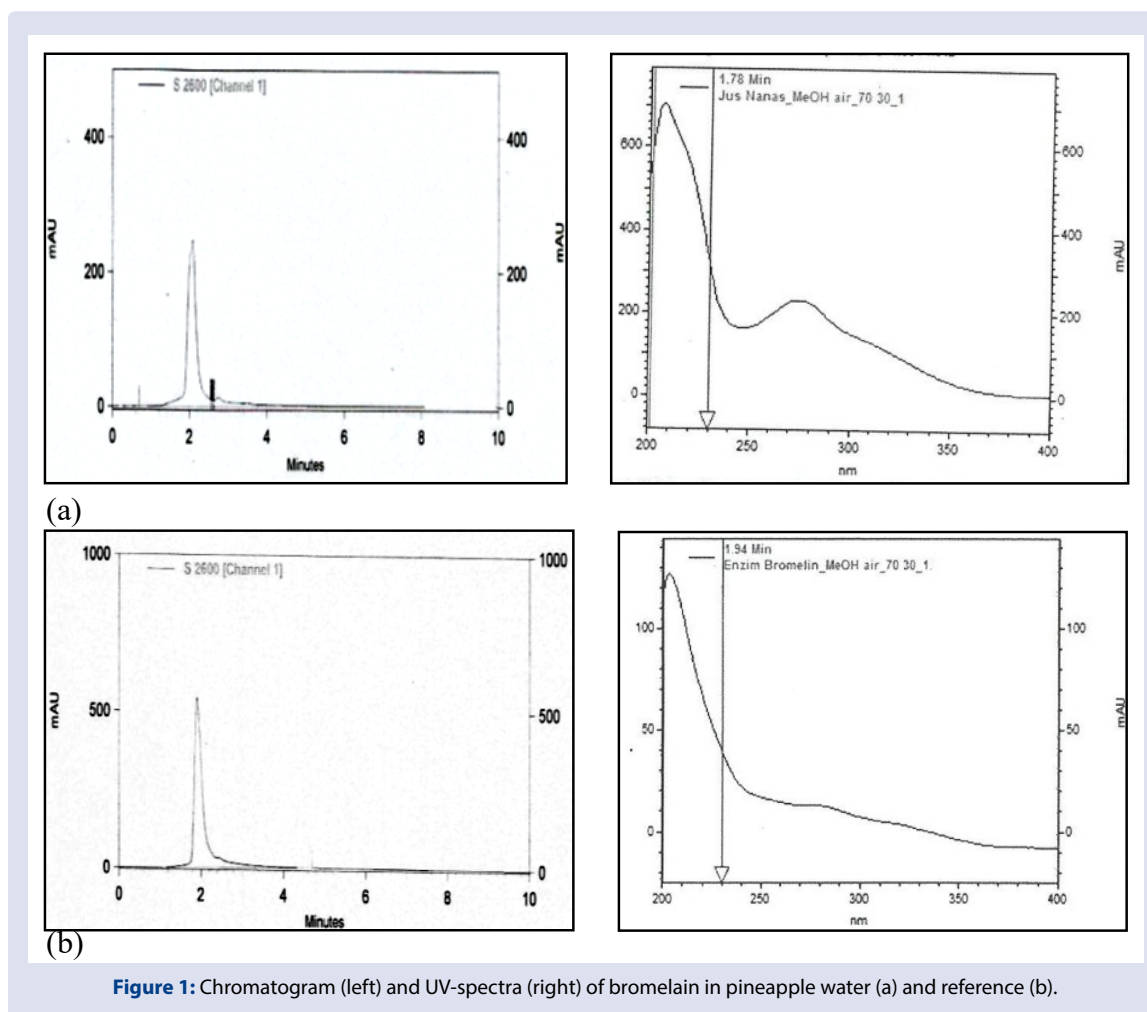
The linearity test is used to describe the correlation of response and the concentrations. Linearity is showed by coefficient determination (R<sup>2</sup>) that closes to 1. The linearity of method was met requirement<sup>18</sup> with R<sup>2</sup> of 0.993 (Figure 2). Thus, it can be concluded that the method produces a linear response to the concentrations of bromelain.

### Accuracy and precision test

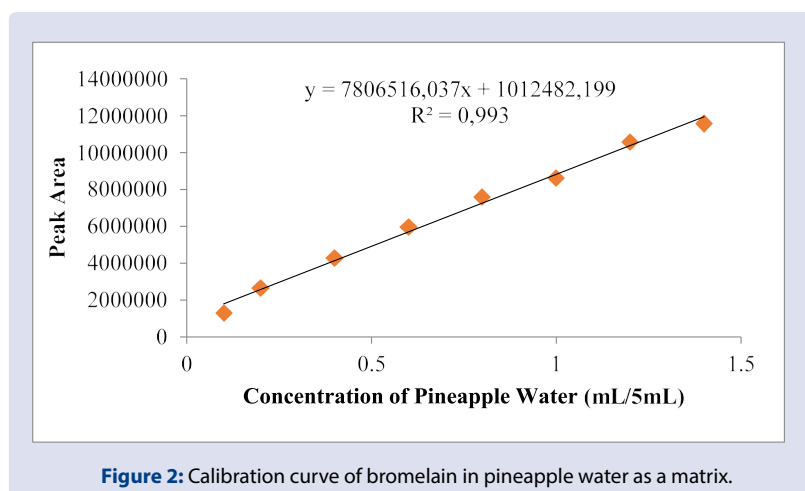
The accuracy and precision tests are carried out by the standard addition method which is to add a reference standard into the sample. This method is chosen because it cannot create a sample matrix without analytes. Reference standard added to the sample is 1 and 2% for efficient use of raw materials. The accuracy of an analytical method describes the closeness of the determined value obtained by the method to the nominal concentration of the analyte (expressed in percentage). The result showed that the recoveries at the addition of 1 and 2% bromelain were 106.37 ± 1.94% and 98.12 ± 1.29% (n = 5), respectively. The precision of the analytical method describes the closeness of repeated individual measures of analyte. Precision is expressed as the coefficient of variation (CV). The result showed that the CV values at the addition of 1 and 2% bromelain were 1.83 and 1.32% (n = 5). This proposed method met the accuracy and precision according to ICH.<sup>18</sup>

### Analysis of bromelain in pineapple fruit water at various storage time

Bromelain level at zero time was 81.53%. Bromelain levels in pineapple water showed decrease after storage for 8 h at 10°C (Figure 3). Bromelain levels in pineapple juice appeared to decrease not statistically significant (p > 0.05) after storage for 8 h at 10°C, with to mean value of 78.46 ± 2.88%. The relative declines in the levels of bromelain were 91.17-99.14% from 1 to 8 h at 10°C (Figure 4).



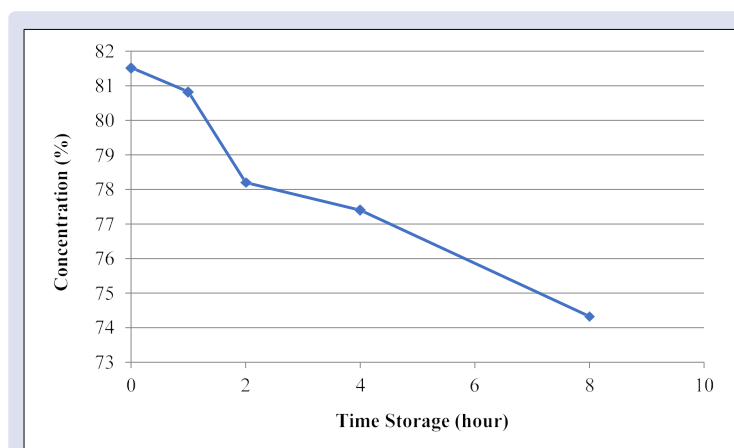
**Figure 1:** Chromatogram (left) and UV-spectra (right) of bromelain in pineapple water (a) and reference (b).



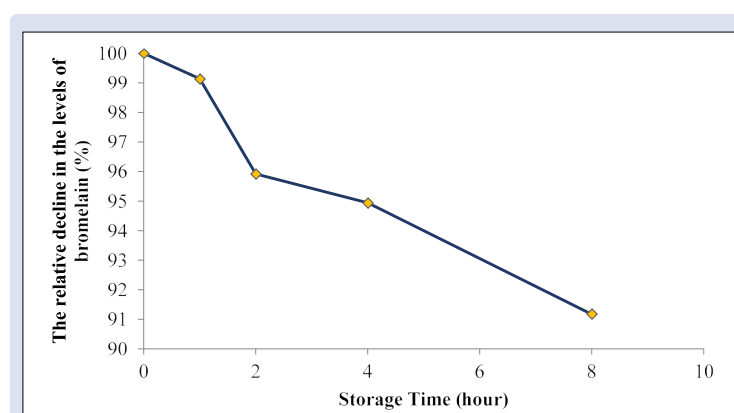
**Figure 2:** Calibration curve of bromelain in pineapple water as a matrix.

**Table 1:** The retention time and peak area of bromelain at various flow rate of mobile phase.

Flow Rate (mL/min)	Retention Time of Analyt (minutes)		Peak Area	
	Reference	Pineapple Water	Reference	Pineapple Water
0.8	2.133	2.300	811943	4900948
1.0	2.167	2.367	929218	4921142
1.2	1.950	1.800	851046	3571425



**Figure 3:** Levels of bromelain at 10°C in 0, 1, 2, 4 and 8 h.



**Figure 4:** The relative declines in the levels of bromelain depend on the storage time.

## CONCLUSION

The currently developed HPLC method was developed, optimized and validated for the analysis of bromelain in pineapple water. It appeared that analysis were supported with C18 (150 mm x 4,6 mm) as stationary phase, methanol-air (70:30) as mobile phase with flow rate of 1.0 mL/min and PDA as a detector at 230 nm. This method is selective, accurate, precise, efficient and simple to determination bromelain level in pineapple water. It was shown to be applicable for the study of shelf life of pineapple water based on bromelain content. With a decrease of 91.17-99.14% from 1 to 8 h at 10°C. Bromelain content in pineapple juice was not statistically significantly ( $\alpha = 0.05$ ) after 8 h storage at 10°C at 2 min.

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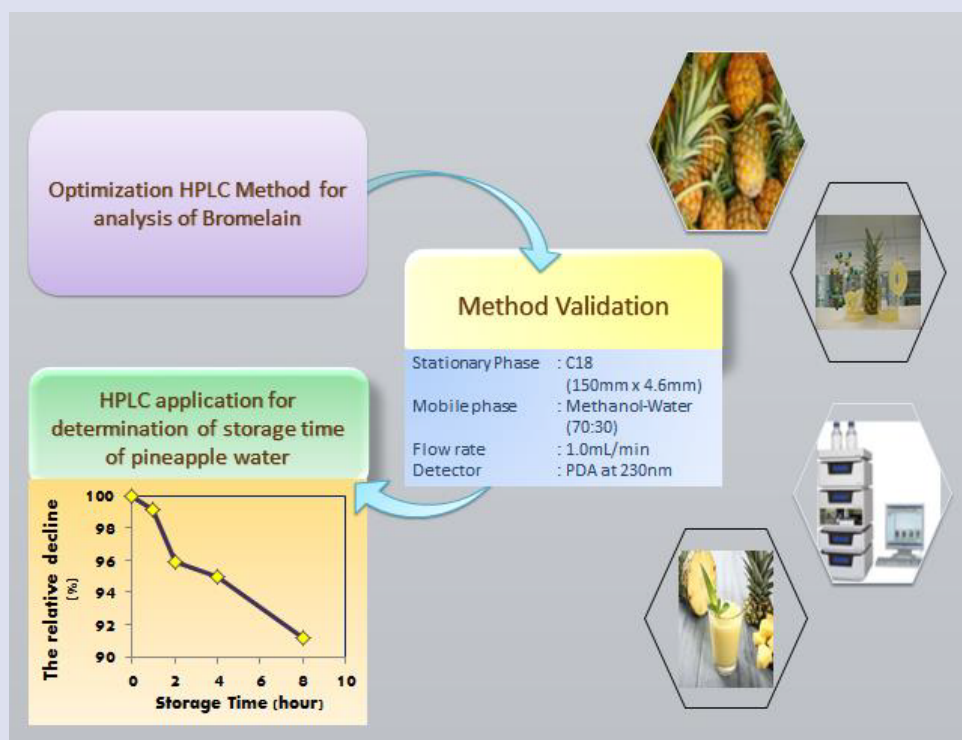
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## GRAPHICAL ABSTRACT



## ABOUT AUTHORS



**Novi Yantih:** Novi Yantih is as an associate professor in Pharmacy Faculty of Universitas Pancasila, Indonesia. She has over 15 scientific papers. She is a recognized expert in areas of analytical chemistry and bioanalysis for pharmacokinetics study and drug discovery. She has graduated her PhD in Faculty of Pharmacy, Universitas Indonesia in January 2018. Recently, she investigated about N-acetyltransferase-2 genetic polymorphisms in Melanesian ethnicity and effect of pineapple as hepatoprotector on INH metabolism. Her contributions in this publication were responsible for optimization and validating of high performance liquid chromatography to determine bromelain for studying of storage time of pineapple water.



**Alfadella Methananda:** Alfadella Methananda was graduated from Faculty of Pharmacy, Universitas Pancasila, Indonesia in July 2016. Her contribution for this publication was helping optimization process and determining bromelain of sample by using high performance liquid chromatography.



**Yahdiana Harahap:** Yahdiana Harahap has completed her PhD at the age of 39 from Department of Pharmacy, Institute Technology Bandung, Indonesia. Now she is the Head of Biavailability and Bioequivalence laboratory Faculty of Pharmacy, Universitas Indonesia. She has published 40 papers published in both International and National Journals. She has been invited to be the speakers in many international conference, especially in the field of Bioanalysis technique. She currently serves as an expert at Indonesia National Agency of Drug and Food Control. For this publication, she supervised the validation process of high performance liquid chromatography to determine bromelain in pineapple water.



**Wahono Sumaryono:** Wahono Sumaryono has completed his Ph.D (Dr.rer.nat) in July 1990, from Institute for Pharmaceutical Biology – Technical University “Carolo –Wilhelmina”, Braunschweig-Fed.Rep.of Germany- Now he is Professor in Natural Product Chemistry at Faculty of Pharmacy Universitas Pancasila Jakarta, since 2007. He served as Dean of the Faculty during Nov 2011 – March 2014, and as Rector of Universitas Pancasila since March 2014. He has published 10 International Scopus journals in the field of Natural Products and related subjects. His contribution for this publication was a supervisor of the extraction and characterization process of bromelain from pineapple.



**Lestari Rahayu:** Lestari Rahayu was a student of Master Degree Program in Faculty of Pharmacy, Universitas Airlangga on 1985-1988. She is an associate professor in Universitas Pancasila. Her research is in the field of pharmacology studies of Natural Products and related subjects. She has published 7 papers in International and National Journals. She was also presented her research in congress/seminar/workshop as oral or poster presentation. She contributed for characterization process of bromelain from pineapple in this publication.

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