# Determination of Gibberellin ($GA_3$) in Liquid biofertilizers

# การหาปริมาณจิบเบอเรลลิน (จีเอ 3) ในน้ำหมักชีวภาพ

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## Abstract

For determination of gibberellin ($GA_3$) content, various fresh fruits (pine apple, shell pine apple, banana, banana shell and young coconut) were extracted with hexane and sequentially purified by liquid-liquid and solid-phase extraction prior to analysis by GC-MS and HPLC. Each kind of fresh fruits was then fermented with molasses in ratio of 3:1(w/w) stored in a plastic bag container at room temperature (28-30$^{o}$C) and monitored for $GA_3$ content in every 15 days intervals. From the GC-MS and HPLC results, the $GA_3$ was found in several fresh fruits such as pine apple, shell pine apple, banana, banana shell and young coconut. In case of fermented fruits, the $GA_3$ was also found in the fermented part obtained from those fruits. The $GA_3$ contents in all fermented fruits treatments were drastically increased as the fermentation time did.

## บทคัดย่อ

การหาปริมาณจิบเบอเรลลิน (จีเอ3) ในผลไม้สดชนิดต่างๆ (สับปะรด เปลือกสับประรด กล้วย เปลือกกล้วย และมะพร้าวอ่อน) ศึกษาโดยนำผลไม้แต่ละชนิดมาสกัดด้วยเฮกเซนและทำบริสุทธิ์ด้วยการสกัดแบบของเหลว-ของเหลวและการสกัดด้วยเฟสของแข็งที่เหมาะสม ตามลำดับ ก่อนจะตรวจวิเคราะห์ด้วยเทคนิค GC-MS และ HPLC จากนั้นทำการหมักผลไม้สดแต่ละชนิดด้วยกากน้ำตาลในอัตราส่วน 3 ต่อ 1 โดยน้ำหนัก โดยหมักใส่ภาชนะพลาสติกปิดฝาเก็บไว้ที่อุณหภูมิห้อง (28-30 $^{o}$C) โดยสุ่มตัวอย่างมาหาปริมาณจีเอ3 ทุกๆ 15 วัน จากข้อมูล GC-MS และ HPLC พบว่าผลไม้สดที่พบจีเอ3 ได้แก่ สับปะรด เปลือกสับประรด กล้วย เปลือกกล้วย และ มะพร้าวอ่อน ในส่วนของน้ำหมักสามารถตรวจพบจีเอ3 เช่นกัน จากการหมักผลไม้ดังกล่าว และยังพบว่าเมื่อใช้เวลาหมักนานขึ้นจะพบจีเอ3 ในปริมาณมากขึ้นตามไปด้วย

**Keywords**: gibberellin ($GA_3$), GC-MS, HPLC

**คำสำคัญ** : จิบเบอเรลลิน (จีเอ3) GC-MS HPLC

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## Introduction

Successful agriculture activities are depend on careful planning. At present, the agricultural system can be divided into two major systems; conventional and organic agricultural system. Many inputs used in conventional chemicals for agricultural purposes are toxic to soil microbes, beneficial insect, and soil invertebrates. Nowadays, several countries have been interested in an organic agriculture system (such as using organic fertilizer) because this system will increase quality of any agriculture system in long run, improving food quality, safety and environmentally better nutrient supplies. Beneficial of the fermented organic fertilizers will likely depend upon the organic fraction, direct effects of the introduced microorganisms, and indirect effects of microbial-synthesized metabolites (e.g. phytohormones). Jiang *et al*., 2006, reported that the plant hormones play vital role in organic agriculture system for plant growth and development. Therefore, identification and determination of these active compounds in organic substances which were used instead of synthetic chemicals and their consequence were needed to improve.

Gibberellins (GAs) are one of the major groups of phytohormone which play important role in specific biological activity in very low concentration and the well known type was $GA_3$. This compound can be produced by higher plants, fungi, bacteria and mosses (Srivastava *et al*., 2003; Unyayar *et al*., 1996) and usually translocated within the plant from a biosynthesis site to an active site. $GA_3$ determination is usually preceded by extensive purification involving, for example liquid-liquid extraction (Schmelz *et al*., 2004), solid phase extraction (Rachev, 1997) or HPLC purification (Joo *et al*., 2005; Castillo and Martinez, 1997). Recently, several techniques have been developed for $GA_3$ determination such as spectrophotometric, CE, HPLC and GC-MS (Castillo, G. and S. Martinez. 1997; Jiang *et al*., 2006). Among the methods available to quantify $GA_3$, mass spectrometry is the most powerful due to its high selectivity and sensitivity. Therefore, in the present studies, the $GA_3$ contains in fresh fruits and liquid biofertilizer were determined using gas chromatography coupled to mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC).

## Materials and Methods

### 1. Chemicals and Plant Materials

The standards: $GA_3$ was obtained as gibberellic acid, 90% (Acros Organics, USA). Methanol (HPLC grade) was purchased from Lab Scan (Ireland). All other chemicals were of analytical reagent. Deionized water used throughout the study was employed from Simplicity Water Purification System (Millipore Corporation, USA). All fruits (coconut, pineapple and banana) and sugarcane molasses (Mitr phol Sugar Corp, Ltd.) used in this experiment were purchased from a local market at Khon Kaen province, Thailand.

Liquid biofertilizers used in this experiment were prepared each kind of fresh fruits was crushed and mixed with molasses in the ratio of 3:1 (w/w). After that, these mixtures were stored in plastic bag containers with lid at room temperature (28-30$^{o}$C).

### 2. Extraction and Purification Procedures

The individual of fresh fruits was chopped to be small pieces and then extracted with hexane three times to ensure the complete extraction of the chemical and bioactive constituents and organic layer was then discarded. The aqueous phase was adjusted pH to be 2.5 with 1M NaOH or 1M HCl. The adjusted pH samples were extracted with ethyl

acetate for three times and the combined of phase extracts were concentrated under reduce pressure. After that, the extractants were then further purified by Oasis MAX cartridges (Waters Associate, USA) prior to analyze by GC-MS and HPLC.

**3. GC-MS conditions**

The GC-MS system used in this study was a Thermo Finnigan (Polaris Q) Ion Trap mass spectrometer. The capillary GC column used was a ZB-5 (30 m x 0.25 mm i.d., Zebron). Conditions were as follow: column temperature was held at 150$^{o}$C for 2 min and then increased at 20$^{o}$C/min to 280$^{o}$C and maintained for 2 min; helium was used as carrier gas at a linear flow of 1 ml/min. Injections were performed using PAL autosampler. The samples were injected in ethanol. Injection and interface temperatures were respectively at 250$^{o}$C and 280$^{o}$C. Electron energy was 70 eV. Data were acquired and processed by Xcalibur software. The identification of the analyze compounds was accomplished by comparing their mass spectra with those of authentic compounds available from computerized spectral database (NIST).

**4. HPLC conditions**

The chromatographic analysis was performed on a Waters liquid chromatograph (Waters, USA). It consists of a Waters 600E Multisolvent Delivery System, a Waters In-Line Degasser AF, a Rheodyne injector with sample loop of 20 µL, a Waters 2996 photodiode array detector, a Waters 2475 multi wavelength fluorescence detector and a Waters temperature control system. Empower software was used for data acquisition.

The effect of mobile phase pH on the retention behavior was studied in pH range of 3.0-7.0. The mobile phase used in this experiment was acetonitrile-water in ratio of 25:75 (v/v). The Nucleosil $C_{18}$ column (5 µm particle size, 150 x 4.6 mm i.d.) from Phenomenex (Australia) was used as an analytical column. The column was equilibrated with mobile phase condition for 30 min. The separation was carried out by isocratic elution with a flow rate of 0.8 mL/min. An injection volume of 20 µL was used for each analysis.

The $GA_3$ standard solution was detected at 208 nm. Capacity factor was calculated from $k= (t_R-t_0)/t_0$, where $t_0$ was the hold-up time, $t_R$ was the retention time of $GA_3$ for each mobile phase. The retention times and capacity factors of the solutes were determined from 6 different injections. Peak identification was based on retention time and spiking of the standard.

Calibration curves were prepared from the stock of $GA_3$ standard solution in the concentration range of 10-100 µg mL$^{-1}$. Calibration standards were prepared with concentrations of 10, 20, 40, 60, 80 and 100 µg mL$^{-1}$. QC standards (n=5) were prepared at 20 and 50 µg mL$^{-1}$. The validation of the developed method was also studied. Intra-day studies were performed by analysis of five replicate QC standards at 20 and 50 µg mL$^{-1}$ during 1 day and inter-day studies were performed by analysis of these same five replicate QC standards on 3 different days (within 1 week). The intra-day studies were designed to determine the stability of the method for a single analysis. The inter-day studies were designed to evaluate the stability of the method for a number of days of analysis.

**Results and Discussion**

**1. Analysis of $GA_3$ in liquid biofertilizers by GC-MS**

The presences of $GA_3$ in the purified samples were successfully screened. Under optimum GC-MS conditions quantitation was performed by injection samples in GC-SIM mode because many compounds could present the same nominal molecular mass. Thus, the combination of the parent mass and unique fragment ions was used to monitor selectively $GA_3$. From the GC-MS results, the $GA_3$ was found in several fresh fruits such as pine apple, shell pine apple, banana, banana shell and young coconut in the range

of 20-50 µg $mL^{-1}$. In case of fermented fruits, the $GA_3$ was also found in the fermented part obtained from those fruits in range of 50-200 µg $mL^{-1}$. The $GA_3$ contents in all fermented fruits treatments were drastically increased as the fermentation time did (Datas not shown). Representative GC-MS chromatograms of $GA_3$ ($T_R$ at 9.22) obtained from standard and water-fermented fruits were shown in **Fig 1**.

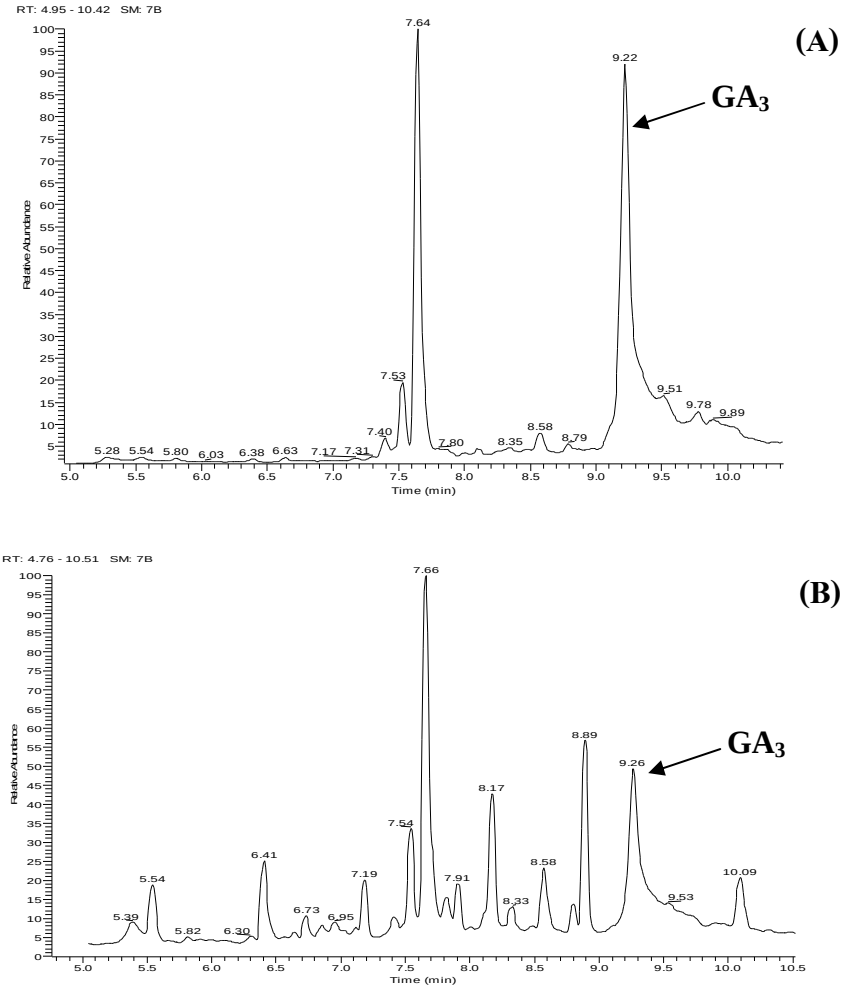



**Fig 1**. GC-MS chromatograms of $GA_3$ **(A)** standard 50 µg $mL^{-1}$ and **(B)** Liquid biofertilizers from coconut at 30 days ; respectively

**2. Analysis of $GA_3$ in liquid biofertilizers by HPLC**

To verify the influence of mobile phase pH on retention behavior, performed experiments using mobile phases containing 25% acetonitrile in water (v/v) with different pH values (3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00 and 7.00) were investigated. As can be seen from **Fig.2**, the retention factor values, k, of $GA_3$ decreased with the increase of pH from 3.0-7.0. The reason of this behavior was that, $GA_3$ contains carboxylic group and its retention depend on the percentage of ionized and non-ionized species (Castillo, G. *et al.,* 1997). Thus, taking account of the analytical time, we used a pH of 4.50 for further experiments. Calibration curve was constructed in the range of 10-100 µg $mL^{-1}$ with six concentrations, each in five replicates, for 3 separate days. The regression equations of this curve and its correlation coefficients ($r^2$) were calculated as follows: $GA_3$, Y = 19234x-82100 ($r^2$ =

0.9984), where Y and X are the peak area and the concentration (μg $mL^{-1}$) of the analytes, respectively.

The method was validated for reproducibility of the retention time and the peak area of the analytes. The relative standard deviations (RSD) of the retention time and the peak area of each peak for six replicate injections were 0.10-0.30% and 1.05-4.40%, respectively. For the same sample, containing $GA_3$ 50 μg $mL^{-1}$, the intra-day reproducibility (n = 5) of peak area of $GA_3$ was lower than 6.70% of RSD, which indicated that the analyte in solution and during the actual analysis was very steady. The detection limits (S/N = 3) and the limit of quantification, LOQ, (S/N = 10) of $GA_3$ were 2.00 and 3.50 μg $mL^{-1}$, respectively. Recovery experiments were performed six replicates adding a 20 μg $mL^{-1}$ of aliquot $GA_3$ into the extracted samples from liquid biofertilizer. The recovery of $GA_3$ was 91.92%. The method also showed good precision.

The $GA_3$ contents in all fermented fruits treatments were drastically increased as the fermentation time did. From HPLC chromatograms of $GA_3$ ($T_R$ at 5.117) obtained from standard and water-fermented fruits were shown in **Fig 3**.

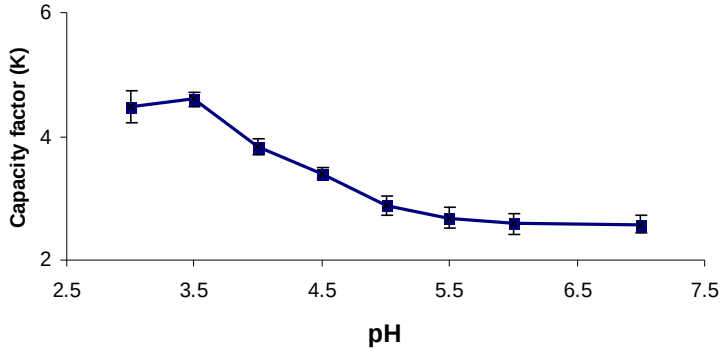
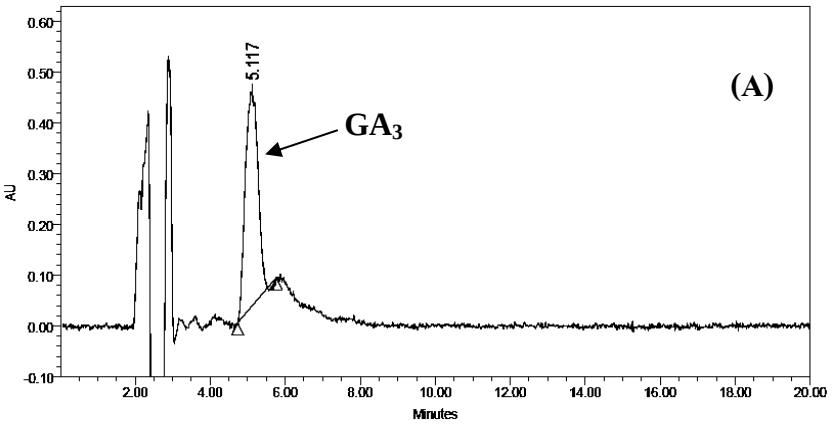



**Fig 2.** The influence pH of mobile phase on retention time.



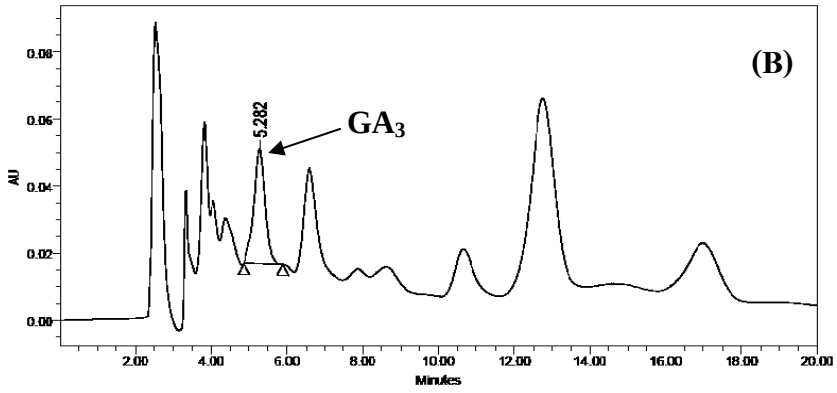



**Fig 3.** HPLC chromatograms of **(A)** $GA_3$ standard 20 µg $mL^{-1}$ **(B)** Liquid biofertilizers from coconut at 30 days

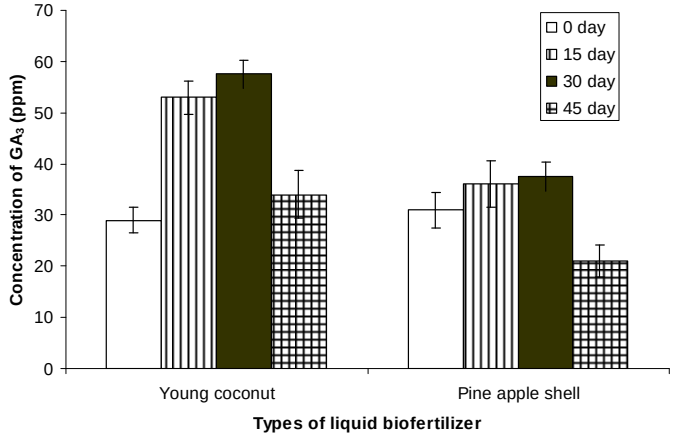



**Fig 4.** Changes in $GA_3$ concentration in liquid biofertilizer prepared from fermented coconut and pine apple shell during fermented time

The amounts of $GA_3$ were analyzed and the results showed high variable of $GA_3$ from different batches of productions **(Fig 4.)**. From chromatograms of GC-MS and HPLC, The levels of $GA_3$ in liquid biofertilizers were five to ten orders of magnitude higher than those observed in fresh fruits. The results indicated that the variation of quantity was depended on the type of fruits. The amount of $GA_3$ in liquid biofertilizers were increased from fresh fruits may be due to microbial activities (Srivastava *et al*., 2003; Unyayar *et al*., 1996).

**Conclusions**

The proposed methods, GC-MS and RP-HPLC, promised to be applicable to the plant hormone analysis both in fresh fruits and liquid biofertilizers. RP-HPLC has become popular for the plant hormone analysis because of its simplicity, rapidity and reliability and GC combined with MS is also very sensitive method for $GA_3$ analysis. Based on the results, the amount of $GA_3$ liquid biofertilizers studied depends on the fruit type which may be due to the composition of compounds in each fruit. It is concluded that the liquid biofertilizer may be a valuable fertilization resource in organic agriculture. They may be used to promote growth of plants and help farmers to

reduce cost due to this product can be simple preparation and would be prepared from local plants.

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