การแยกและวิเคราะห์ปริมาณกรดแกลลิกและกรดเอลลาจิก ด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง

Separation and Determination of Gallic Acid and Ellagic Acid by High Performance Liquid Chromatographicmethod

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บทคัดย่อ

ได้เตรียมเฟสคงที่พอลิเมอร์ลอกแบบโมเลกุลสำหรับแยกกรดแกลลิก(จีเอ) และกรดเอลลาจิก (อีจีเอ) เตรียมพอลิเมอร์ด้วยวิธีให้ความร้อนที่ประกอบด้วยเบนโซอิลเปอร์ออกไซด์เป็นตัวเริ่มปฏิกิริยา ตัวทำละลายผสมระหว่างโดเดคคานอล และทูลูอีนไดไวนิลเบนซีนเป็นตัวเชื่อมโยงโมเลกุลและสไตรีนเป็นมอนอเมอร์ตามลำดับ ทำการแยกกรดแกลลิกและกรดเอลลาจิกด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง โดยใช้คอลัมน์ GA-EGA-MIP ขนาด 50×4.6 มิลลิเมตรแยกด้วยเฟสเคลื่อนที่ระหว่างน้ำและอะซิโตไนไตรล์ ที่พีเอช 3 (อัตราส่วน 5:95 โดยปริมาตร) อัตราการไหล 0.1 มิลลิลิตรต่อนาที และตรวจวัดที่ความยาวคลื่น 280 นาโนเมตร เก็บสารละลายตัวอย่างจากที่แยกได้จากคอลัมน์ GA-EGA-MIP และนำมาวิเคราะห์หาปริมาณกรดแกลลิกและกรดเอลลาจิกในตัวอย่าง ที่ถูกแยกด้วยคอลัมน์ Ultra C18 ขนาด 150×4.6 มิลลิเมตรแยกสารโดยมีการปรับอัตราส่วนของอะซิโตไนไตรล์และน้ำในช่วงเวลาการแยกด้วยอัตราการไหล 1.0 มิลลิลิตรต่อนาที ปริมาตรฉีดสาร 10 ไมโครลิตร และตรวจวัดที่ความยาวคลื่น 280 นาโนเมตร พบขีดจำกัดของการวิเคราะห์ได้ของกรดแกลลิกและกรดเอลลาจิก เท่ากับ 1.02 และ 2.29 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ วิธีนี้มีความเหมาะสมสำหรับแยกและทำให้บริสุทธิ์ของกรดแกลลิกและกรดเอลลาจิกจากสารสกัดลำไย

คำสำคัญ : พอลิเมอร์ลอกแบบโมเลกุล / โครมาโทรกราฟีของเหลวสมรรถนะสูง / กรดแกลลิก / กรดเอลลาจิก / ลำไย

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Abstract

Molecularly imprinted polymers stationary phase was prepared for the separation of gallic acid (GA) and ellagic acid (EGA) by thermal polymerization method using benzoylperoxide as initiator, the mixtures of dodecanol and toluene as porogens, divinylbenzene as cross-linker and styrene as a functional monomer, respectively. Gallic acid and ellagic acid solutions were separated by HPLC using GA-EGA-MIP column, 50×4.6 mm as MIP column with the mobile phase; water-acetonitrile (5:95, v/v) adjusted to pH 3.0. The flow rate was adjusted to 0.1 mL min^{-1}. The absorption was made at 280 nm. The sample solution was collected from GA-EGA-MIP column and then was injected into the analytical column used was Ultra C18 column, 150×4.6 mm for determination of gallic acid and ellagic acid in sample solution. The mobile phase was eluted with gradient system containing a mixture of acetonitrile and water. The flow rate was used to 1.0 mL min^{-1}. The injection volume was adjusted to 10 μL and the absorption was made at 280 nm. The results of quantitation limit of GA and EGA were found to be 1.02 and 2.29 μg mL^{-1}, respectively. This approach method is shown to be successfully for the separation and purification of gallic acid and ellagic acid from *Dimocarpus longan* extract.

Keywords : Molecularly imprinted polymers, High performance liquid chromatography, Gallic acid, Ellagic acid, *Dimocarpus longan*

1. Introduction

Longan (*Dimocarpus longan* Lour.) is classified in family Sapindaceae. The Edor, longan cultivar, is the mostly cultivated and consumed in Northern Thailand including Chiang Rai and Chiang Mai province. In 2011, the cultivated yield of Longan was approximately 47,580 tons in Chiang Rai and Chiang Mai provinces. For the fruit industrial processing and fresh consumption, these seeds become as the by-product (~10-15% w/w of whole fruit). Longan seed and peel have previously been shown to possess potent antioxidant activities which could be ascribed to their phenolic contents such as gallic acid. Gallic acid (GA) is known to have anti-inflammatory, antimutagenic, anticancer and antioxidant activity (Jiang *et al*., 2002; Lin *et al*., 2005; Matsumoto, 2006). Various methods have been developed for determining gallic acid, including chromatographic methods (Amakura *et al*., 2000; Garcia del Moral *et al*., 2007). Its dimeric derivative, known as ellagic acid (EA), exists either in the free form or bound as gallo-(GT) and ellagitannins (ET), respectively. These hydrolyzable tannins (HTs) are presented with

rich varieties of plants and are presented in tea, red wine, fruits, beverages and various medicinal plants.The technique of molecular imprinting polymerization (MIP) is introduced in 1972. The pre-organized approach, mainly developed by Wulff (1995) where the aggregates in solution prior to polymerization is maintained by covalent bonds and the self-assembly approach, mainly developed by Mosbach and Ramstrom (1996) where the pre-aggregates between the print molecule and the functional monomers are formed by non-covalent or coordination interactions. Molecularly imprinted polymers are extensively cross-linked polymers containing specific recognition sites with a predetermined selectivity for analyses of interest. The technique involves complexation in a solution of target molecules (template) with functional monomers through either covalent or non-covalent bonds, followed by a polymerization reaction by an excess of cross-linkers. Removal of the templates leaves behind specific recognition sites that are complementary to the template in terms of its shape, size and functionality in the polymer network. These recognition sites enable imprinted polymers to be used as the mimics of enzymes, receptors and antibodies for screening various kinds of compounds from a mixture with abundant interferences. Up to now, there have been reviews summarizing the development of MIP (Janssen *et al.,* 1975; Kandimalla & Ju, 2004; Ye & Haupt *et al*., 2004), which have covered many aspects from the sorbents for sample preconcentration and stationary phase for separation to bioassays, biosensors and mimics for enzymes, receptors and catalysts. The molecular imprinting technique which combines the advantages of tailor-made sorbents and physical durability is only one way to solve this problem. This technique has been widely used in the production of molecularly imprinted polymers (MIPs) with specific binding sites for a wide variety of molecules (Rathbone, 2005; Thongchai *et al*., 2010). Furthermore, usually MIPs is reusable, need low cost of preparation; exhibit a high mechanical and chemical stability. A relatively new development in the area was used MIPs for sample clean-up and sensor determination.

Thus, the aim of this research was investigated to preparing MIP for separate and purify of gallic acid and ellagic acid and then using HPLC determination of gallic acid and ellagic acid in longan extract. Moreover, the utilization to quality control of fruit processing industry by-products as a material for value-added product preparation would be useful.

2. Materials and Methods

Molecularly imprinted polymer preparation

The stationary phase was directly prepared by in situ polymerization within the stainless steel chromatography column tube of 50×4.6 mm i.d. A prepolymerisation solution consisting of 0.029 $mmolL^{-1}$ of gallic acid and 0.017 $mmolL^{-1}$ of ellagic acid, 250 μL DVB as the cross-linking agent, 150 μL styrene as a functional monomer and 420

μL dodecanol, 180 μL toluene as porogenic solvents, 37 mg BP as initiator were prepared in a screw-capped glass vial. The molar ratio of the template for the preparation MIPs was 1:2. The MIP solution was sonicated for 20 min, and then purged with a stream of nitrogen for 10 min. Approximately 1.0 mL of the reaction solution is flushed through the stainless steel chromatography column tube of 50×4.6 mm i.d. to wet thoroughly the wall surface before filling the column with the solution. The filled column has been thermal initiation at 65 ^{o}C for 24 hours in hot air oven. After thermal initiation, the column is washed with ethanol and acetonitrile using a hand-held syringe to remove unreacted reagents (GA-EGA-MIP column). Non-imprinted polymers (NIPs) were prepared simultaneously under the same conditions without the addition of the template (Non-MIP column). Microscopic analysis of the MIP column was performed in Leo1455VP Scanning Electron Microscope.

Apparatus and instruments

(1) Online solid phase extraction by GA-EGA-MIP column: The chromatographic system for the separation and analysis of gallic acid and ellagic acid in longan extracts were carried out with Shimadzu Model SCL-10A liquid chromatography, thermostatic column compartment, online degasser and an UV-visible detector model SPD-10A. The monolithic separation column used was GA-EGA-MIP (50×4.6 mm i.d.) before analysis. Mobile phase was a mixture containing varying ratios of acetonitrile and water. The flow rate was adjusted to 0.1 mL min^{-1}. The injection volume was adjusted to 10 μL and the absorption was made at 280 nm. The sample solution was prepared and vacuum-filtered through 0.45 μm nylon membrane before use. The following instruments were also used; simultaneous spectrophotometer (UV mini-1240, Shimadzu) was used to scan the spectra of gallic acid and ellagic acid, pH-meter (Model pH 900, Precisa); Switzerland, water bath and shaker (Model SB-200-10); Thailand, Ultrasonicator (Model 889, Cole Parmer); USA and a rotary evaporator (EYELA N-N series); polytron (PT-MR 3000). The adsorption capacity, being the value of the amount of adsorbed substance obtained in a saturated solution was calculated by using equation (1): adsorption capacity = adsorbed (μg) / weight of polymer added (mg) (IUPAC; 1997).

(2) Quantification by RP-HPLC: The sample solution (from online solid phase extraction by GA-EGA-MIP column) was injectedinto the analytical column used was Ultra C18 column, 5 mm, 150×4.6 mm for determination of gallic acid and ellagic acid in the sample solution. The mobile phase was eluted with a gradient system containing a mixture of acetonitrile and water (Table 1). The flow rate was adjusted to 1.0 mL min^{-1}. The injection volume was adjusted to 10 μL and the absorption was made at 280 nm

Standard solutions

The stock standard solutions of gallic acid and ellagic acid were prepared in acetonitrile to provide concentrations of 1,000 mg L^{-1}. These stock solutions were freshly prepared each time and stored below 4°C and protected from light. These solutions were diluted with acetonitrile to the desired concentration levels just before performing the analysis.

Sample and sample pre-treatments

Longan samples were purchased from commercial sources in Chiang Mai and Phitsanulok provinces, Thailand. The sample was collected in fresh and dried in hot air oven at 50°C for 24 hours. The dried material (seed and peel) was ground to a fine powder and kept in an air-tight container at 4°C until further use. The powder sample (100 g) was extracted with ethanol by soxhlet extraction process for 3 hours. The organic solution was evaporated to dryness at 60°C by mean of a rotary evaporator (Buchi, Switzerland). The 10 mg of crude extract was transferred into a 5 mL volumetric flask and made up to volume with acetonitrile. An aliquot of this solution was filtered through a 0.45 μm nylon membrane. Then 10 μL of this solution was injected into the HPLC system for analysis of gallic acid and ellagic acid.

3. Results and Discussion

Preliminary study

The synthesis method for the preparation of the MIP was adapted from that described by Schirmer *et al*. (2006). The ability of the MIP to trap the gallic acid and ellagic acid were initially evaluated in bulk using UV detection where the styrene as the functional monomers. These results show the comparison to the polymer obtained without the incorporation of the template (NIP). It can be seen from Figure 1 that much better enrichment is seen with styrene-MIP which shows the adsorption capacity. Figure 2 shows scanning electron micrographs for the styrene-MIP, these show a porous surface with pore diameter distributions from 2-5 mm compared with NIP. The effect of backpressure was studied by according to theory for conventional chromatographic system;the column length largely affects the separation of analytes. In addition, an advantage by using a short column was that it exhibited a lower resistance to flow rate than the long column. Backpressure on a short column with a length of 50 mm was only 265 kgf at the flow rate 0.1 mL min^{-1}, whereas was only 406 kgf on a long column with a length of 100 mm at the same flow rate.

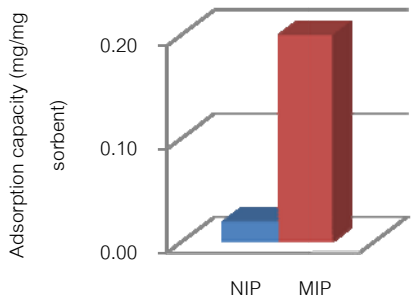

Figure 1 The effect of adsorption capacity comparison between styrene-MIP and NIP

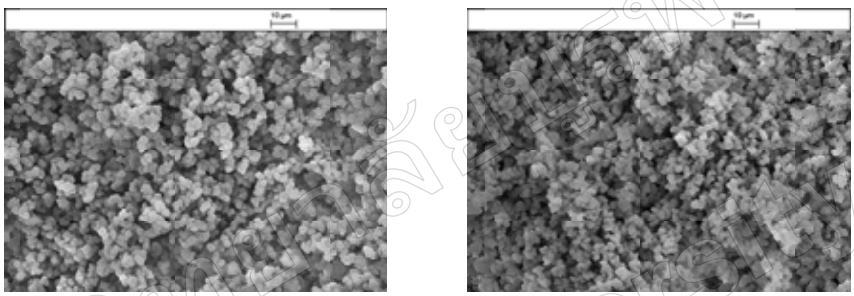

Figure 2 Scanning electron micrographs of the MIP and NIP by thermal-polymerization method

Separation method

A precursory experiment was carried to investigate the spectral characteristics of gallic acid and ellagic acid. The absorption spectrum was studied by spectrophotometer. Themaximum absorption spectrum was obtained by scanning the wavelength over the range of 200-400 nm. The UV spectrum of gallic acid and ellagic acid standards showed the absorption maxima at 280 nm. The sample separation was performed at a flow rate of 0.1 mL min^{-1} and using GA-EGA-MIP column, 50×4.6 mm as a separation column with the mobile phase water-acetonitrile (5:95, v/v) adjusted to pH 3.0. The eluent solution was collected into the test tube from 20-80 minute (Fig. 3). Then this sample solution was determined by HPLC using Ultra C18 column, 5 µm, 150x4.6 mm (Fig. 4).

Table 1 The gradient elution profile of mobile phase

Time (min)	Milli-Q water (%)	Acetonitrile (%)
0.01	90	10
10.00	60	40
20.00	20	80
22.00	60	40
30.00	60	40

Table 2 Intra- and inter day for the studied gallic acid and ellagic acid standard solutions (n=7)

Compounds	concentration ($\mu g\ mL^{-1}$)	Intra-day precision	Inter-day precision
		% R.S.D	% R.S.D
Gallic acid	5.0	1.34	1.02
	10.0	1.81	1.00
	15.0	1.48	1.01
Ellagic acid	5.0	1.34	1.02
	10.0	1.81	1.00
	15.0	1.48	1.01

Table 3 Analytical recovery of gallic acid and ellagic acid added to longan sample solution

Longan sample	% Recovery	
	Gallic acid	Ellagic acid
Longan seed	97.01	95.08
	98.12	96.15
	94.08	94.98
Mean±S.D.	96.40±2.08	95.40±.0.64
Longan peel	78.07	88.59
	86.11	86.13
	96.22	96.87
Mean±S.D.	86.79±9.08	90.53±5.62

Table 4 Gallic acid and ellagic acid contents in longan sample(Edor)

Sample	Content	
	Gallic acid (μg/g)	Ellagic acid (μg/g)
Longan seed	35.41	9.49
Longan peel	60.37	31.43

Method validation

Linearity

Under the selected chromatographic conditions, the linearity of calibration graph was determined using the optimal experimental parameters. Three standard solutions ranging from 5, 10, 15 and 20 μg mL^{-1} were injected into the HPLC system. The calibration graph was obtained by plotting the area under the peak of the solutions against the standard concentrations. Linear calibration graph over the concentration range of gallic acid and ellagic acid were shown with correlation coefficient more than 0.99.

Limit of quantification

Limit of quantification (LOQ) of GA and EGA was estimated from the calibration curve using the expression 10SD/S where SD is the standard deviation of the blank (or the intercept of the calibration curve) and S is the slope of the calibration curve. The quantitation limit (10σ) of GA and EGA were found to be 1.02 μg mL^{-1} and 2.29 μg mL^{-1}, respectively.

Precision

The precision of the method was determined by measuring the repeatability (intraday precision) and the intermediate precision (inter day precision), both expressed as the relative standard deviation (R.S.D). The precision was evaluated by assaying six replicate injections of 5, 10 and 15 μg mL^{-1} of gallic acid and ellagic acid standard solutions, respectively. The repeatability was evaluated each sample on the same day under the same experimental conditions. The intermediate precision was evaluated by assaying each sample on three different days. The results of repeatability and intermediate precisions are shown in Table 2.

Accuracy

Accuracy of the method was assessed with recovery using the addition of three known concentration levels. All samples were injected in three replicates for each concentration. The concentration found was calculated against the concentration added (Table 3). Additives and excipients did not interfere with the determination of

those active ingredients since the samples used to evaluate recoveries were prepared with those additives and excipients present.

Application

The method has been successfully applied to the determination of gallic acid in Longan samples as shown in table 4. The sample was separated using GA-EGA-MIP, 50×4.6 mm. The sample solutions were collected for determination of gallic acid and ellagic acid by reverse phase HPLC method. As shown by HPLC chromatograms of MIP and NIP sample (Fig. 4 and 5), GA and EGA were more retained from MIP than NIP as expected in relation to the selectivity of the imprinted polymers toward the template. Figure 3 reported the chromatogram of the eluting media, showing the selectivity of GA and EGA.

4. Conclusion

In this work, a simple, selective and highly sensitive MIP-LC system has been developed for the analysis of gallic acid and ellagic acid. This simple instrumentation is low flow rates and can be used few reagents. The GA-EGA-imprinted polymer was prepared with thermal polymerization on the column. The synthesis of polymerization consisting of 0.029 $mmolL^{-1}$ of gallic acid and 0.017 $mmolL^{-1}$ of ellagic acid, 250 μL DVB as the cross-linking agent, 150 μL styrene as a functional monomer and 420 μL dodecanol, 180 μL toluene as porogenic solvents, 37 mg BP as initiator were collected in this experiment. Gallic acid and ellagic acid solutions were separated by HPLC using GA-EGA-MIP column, 50×4.6 mm as a MIP column with the mobile phase; water-acetonitrile (5:95, v/v) adjusted to pH 3.0. The flow rate was adjusted to 0.1 mL min^{-1}. The absorption was made at 280 nm. The sample solution was collected from GA-EGA-MIP column and then was injected into the analytical column used was an Ultra C18 column, 150×4.6 mm for determination of gallic acid and ellagic acid in the sample solution. The mobile phase was eluted with a gradient system containing a mixture of acetonitrile and water. The flow rate was used to 1.0 mL min^{-1}. The injection volume was adjusted to 10 μL and the absorption was made at 280 nm. The results of the quantitation limit of GA and EGA were found to be 1.02 and 2.29 μg mL^{-1}, respectively. The proposed method was proved to be simple, rapid, selective and sensitive for the quantitative analysis. The method has been successfully applied to the determination of gallic acid in longan samples.

5. Acknowledgements

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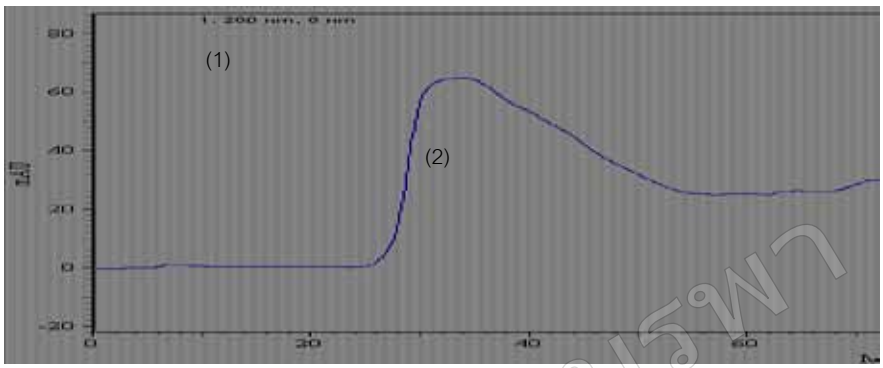

Figure 3 Chromatograms of sample using GA-EGA-MIP column, 50×4.6 mm [gallic acid (1) and ellagic acid (2)]

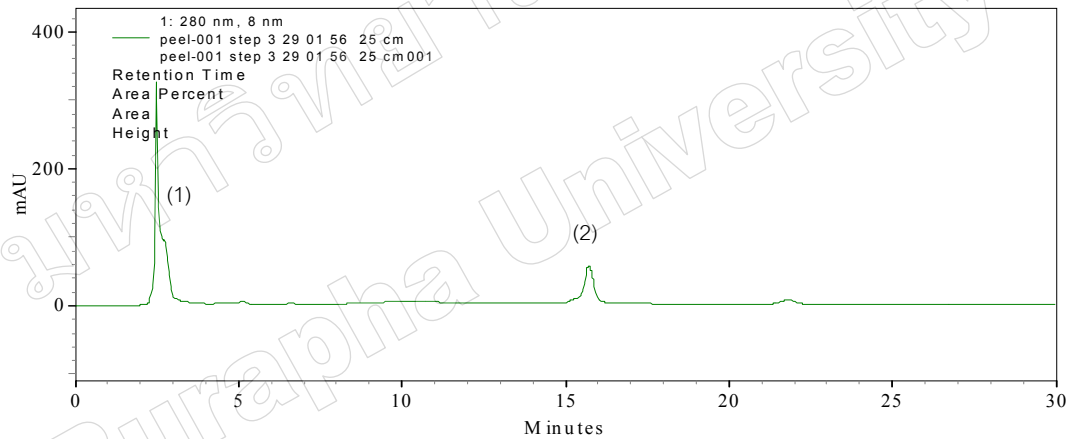

Figure 4 Chromatograms of sample using Ultra C18 column[gallic acid (1) and ellagic acid (2)] after load with GA-EGA-MIP column

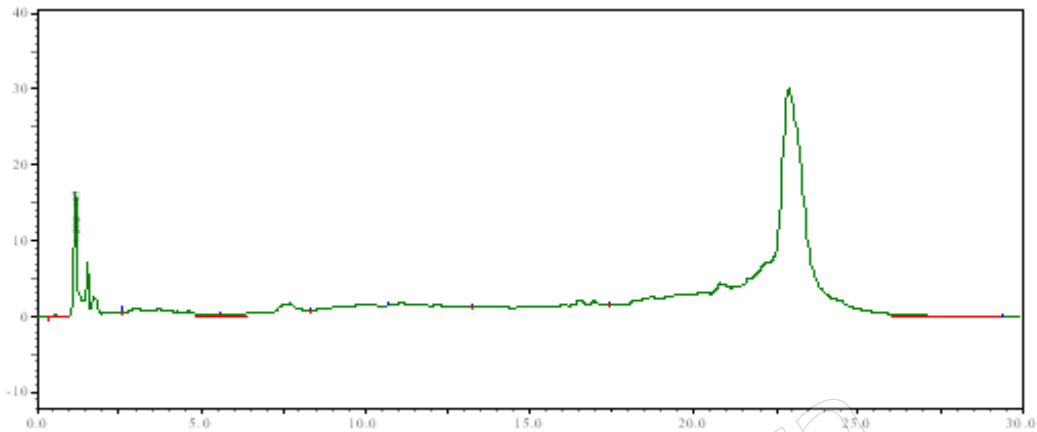

Figure 5 Chromatograms of sample using Ultra C18 column, 5 µm, 150x4.6 mmafter load with Non-MIP column

6. References

Amakura, Y., Okada M., Tsuji S.& Tonogai, Y.J. (2000). Postharvest biologyand handling of longan fruit (Dimocarpus longan Lour.). *Chromatogr. A.*, 896, 87-93.

Garcia del Moral, P., Arin, M.J., Resines, J.A., & Diez, M.T.J. (2007). Determination of ellagic acid in oak leaves and in sheep ruminal fluid byion-pair RP-HPLC, *Chromatogr. B.,* 855, 276-279.

Janssen, G. & Vanderhaeghe, H. (1975). Thin-layerchromatographyof chloramphenicol and thiamphenicol. *J. Chromatogr. A*., 114, 266.

Jiang, Y. M., Zhang, Z.Q., Joyce, D.C., & Ketsa, S. (2002). *PostharVest Biol. Technol.,* 26, 241-252.

Kandimalla, V.B., & Ju, H.X. (2004). A conductive ormosil encapsulated with ferrocene conjugate and multiwall carbon nanotubes forbiosensing application. *Anal. Bio. Chem.*, 380, 587.

Lin, T., Lin, Y., & Ishiki, K. (2005). Genetic diversity of Dimocarpus longan in China revealed by AFLP markers and partial rbcL genesequences.*Sci. Hortic*. 103, 489-498.

Matsumoto, T. K. (2006). Genes uniquely expressed in vegetative and potassium chlorate induced floral buds of Dimocarpus longan. *Plant Sci.,* 170, 500-510.

Mosbach, K. & Ramstrom, O. (1996). The emerging technique of molecular imprinting and its future impact on biotechnology. *Bio. Techno.*, 14, 163.

Rathbone, D.L. (2005). Molecularly imprinted polymers in the drug discovery process. *Adv. Drug. Deliv. Rev*. 57, 1854.

Schirmer C., & Meisel H. (2006). Synthesis of a molecularly imprinted polymer for the selective solid-phase extraction of chloramphenicol from honey. *J. Chromatogr. A*., 1132, 325.

Thongchai, W., Liawruangath, B., Liawruangrath, S., & Greenway, G.M. (2010). A microflow chemiluminescence system for the determination of chloramphenicol in honey with preconcentration using a molecularly imprinted polymer, *Talanta*, 82 (2), 560-566.

Wulff, G. (1995). Molecular imprinting in cross-linked materials with the aid of molecular templates-a way towards artificial antibodies. *Chem. Int. Ed. Engl*., 34, 1812.

Ye, L., & Haupt, K. (2004). Molecularly imprinted polymers as antibody and receptor mimics for assays, sensors and drug discovery. *Anal. Bio. Chem*., 378, 1887.