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ประวัติผู้วิจัย

1. ผู้สมัครขอรับทุน (ชื่อ-สกุล ภาษาไทย) ผู้ช่วยศาสตราจารย์ ดร. วิษณุ ธงไชย

(ชื่อ-สกุล ภาษาอังกฤษ) Assistant Professor Dr. Wisanu Thongchai

2. ตำแหน่งปัจจุบัน

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3. หน่วยงานและสถานที่อยู่ที่ติดต่อได้สะดวก

สาขาวิชาเคมี คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยราชภัฏพิบูลสงคราม
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4. ประวัติการศึกษา

1. ปริญญาตรี สาขาวิชา เคมี คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยราชภัฏพิบูลสงคราม

ปีที่สำเร็จ พ.ศ. 2542

2. ปริญญาโท สาขาวิชา วิทยาศาสตร์เภสัชกรรม คณะเภสัชศาสตร์ มหาวิทยาลัยเชียงใหม่

ปีที่สำเร็จ พ.ศ. 2548
หัวข้อวิทยานิพนธ์ที่ทำ

Development of High Performance Liquid Chromatographic Method for the Determination of Arbutin in Creams and Medicinal Plant Extracts

3. ปริญญาเอก สาขาวิชา เภสัชศาสตร์ คณะเภสัชศาสตร์ มหาวิทยาลัยเชียงใหม่

ปีที่สำเร็จ พ.ศ. 2553
หัวข้อวิทยานิพนธ์ที่ทำ (ทุนโครงการปริญญาเอกกาญจนาภิเษก)

Development of Flow Injection Analysis Methods for the Determination of Bioactive Compounds from Thai Medicinal Plants and Drug Residue

5. สาขาวิชาการที่มีความเชี่ยวชาญพิเศษ

- Medicinal plant and pharmaceutical analysis
- *Flow injection techniques* เช่น *Flow injection analysis, microflow injection analysis,* sequential injection analysis
- Microfluidic system and Lab on a chip

6. งานวิจัยที่ได้รับทุน (หัวหน้าโครงการ)

1. ทุนสนับสนุนการวิจัยจากงบประมาณแผ่นดินของมหาวิทยาลัยราชภัฏพิบูลสงคราม ประจำปีงบประมาณ พ.ศ. 2554 เรื่อง เทคนิคทางโครมาโทกราฟีในการควบคุมคุณภาพและการวิเคราะห์สารเคอร์คูมินอยด์ในวัตถุดิบและผลิตภัณฑ์เครื่องสำอางจากพืชในสกุลเคอร์คูมา
2. ทุนงบประมาณเครือข่ายการวิจัยภาคเหนือตอนล่าง ประจำปีงบประมาณ พ.ศ. 2554 เรื่อง การเตรียมและวิเคราะห์ทางเคมีของน้ำมันข้าวโพดเพื่อพัฒนาเป็นผลิตอาหารเสริมสุขภาพและเครื่องสำอาง
3. ทุนสนับสนุนการวิจัยจากงบประมาณแผ่นดินของมหาวิทยาลัยราชภัฏพิบูลสงคราม ประจำปีงบประมาณ พ.ศ. 2554 เรื่อง การพัฒนาวิธีสกัดและวิเคราะห์สารออกฤทธิ์ทางชีวภาพจากลำไยสำหรับใช้ทางการแพทย์และเครื่องสำอางด้วยเทคนิคโมเลกุลาร์อิมพริ้นเต็ดโพลีเมอร์ร่วมกับแลปออนอะชิพ
4. ทุนสำนักงานคณะกรรมการวิจัยแห่งชาติ ประจำปีงบประมาณ พ.ศ. 2554 เรื่อง การประยุกต์ใช้น้ำมันจากผลอะโวกาโดและพัฒนาเป็นผลิตภัณฑ์เสริมอาหารและเครื่องสำอางนาโนพาร์ติเคิลเพื่อพัฒนาสู่ชุมชน

7. ผลงานวิจัย

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8. ผลงานวิชาการอื่นๆ

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4. **W. Thongchai**, B. Liawruangrath and S. Liawruangrath, High-performance liquid chromatographic determination of arbutin in skin whitening creams and medicinal plant extracts, *The Sixth Princess Chulabhorn International Science Congress*, 2007.
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7. **W. Thongchai**, B. Liawruangrath, C. Chiyasut and P. Leelapornpisid, Determination of Arbutin in Skin-Whitening Cosmetics by High Performance Liquid Chromatography, *The 31st Congress on Science and Technology of Thailand*, 2005.
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17. **W. Thongchai**, B. Liawruangrath, S. Liawruangrath and S. Saysin, High performance liquid chromatographic method for the determination of solasodine in *solanum* species, *PACCON conference*, 2010.
18. **W. Thongchai**, C. Thongpoon, B. Liawruangrath and S. Liawruangrath, LC-MS/MS ON MICROFLUIDIC DEVICE FOR CHLORAMPHENICOL DETERMINATION IN MILK AND HONEY SAMPLES BASED ON MOLECULAR IMPRINTED POLYMERS, ICNST 2012. (Preceeding)

Molecularly Imprinted Polymer for Separation of Gallic Acid and Ellagic Acid

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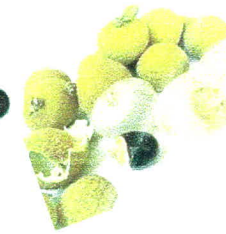
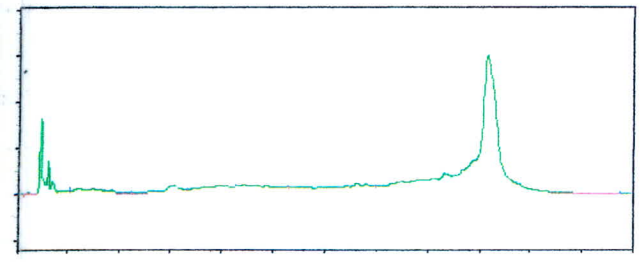
MOLECULARLY imprinted polymers stationary phase was prepared for the separation of gallic acid (GA) and ellagic acid (EGA) by thermal polymerization method using benzoyl peroxide as initiator, the mixtures of dodecanol and toluene as porogens, divinylbenzene as cross-linker and styrene as functional monomer, respectively. Gallic acid and ellagic acid were determined by HPLC using GA-EGA-MIP column, 50×4.6 mm as an analytical 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 injection volume was adjusted to 10 µL, and the absorption was made at 280 nm. This approach method is shown to be successful for the separation and purification of gallic acid and ellagic acid from the extract of *Dimocarpus longan* peels.

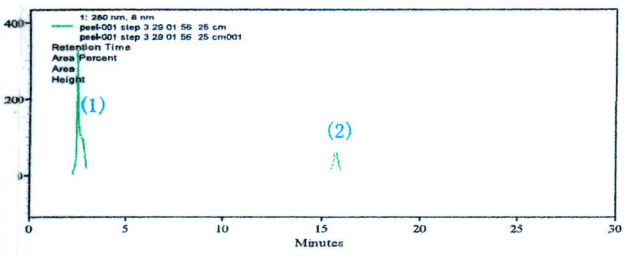


Polymer Preparation

A prepolymerisation solution consisting of 5 mg GA, 250 mL DVB, 150 mL styrene as functional monomer and 420 mL dodecanol, 180 mL toluene, 37 mg BP as initiator were prepared in a screw-capped glass vial. The molar ratio of the template for the prepared MIPs was 1:2. The 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 column (50×4.6 mm) to wet thoroughly the wall surface before filling the column with the solution. The filled column is thermal initiation at 65 °C for 24 h. After thermal initiation, the column is washed with ethanol using a hand-held syringe to remove unreacted reagents. Non-imprinted polymers (NIPs) were prepared simultaneously under the same conditions without the addition of the template.

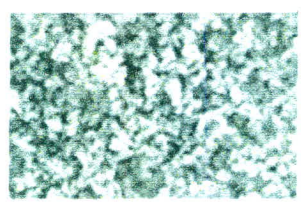


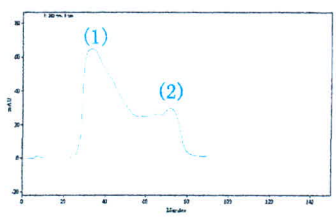


Chromatogram separation of sample using Ultra C18 column [gallic acid (1) and ellagic acid (2)] (A) after load with NIP and (B) after load with GA-EGA-MIP

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 cab be seen from Figure 1 that much better enrichment is seen with styrene-MIP which shows the adsorption capacity.





Scanning electron micrographs of the MIPs by thermal-polymerization method and chromatogram of standard mixture of gallic acid (1) and ellagic acid (2)

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 column. The synthesis of polymerization consisting of 0.029 $mmolL^{-1}$ of gallic acid and 0.017 $mmolL^{-1}$ of ellagic acid, 250 mL DVB as the cross-linking agent, 150 mL styrene as functional monomer and 420 mL dodecanol, 180 mL toluene as porogenic solvents, 37 mg BP as initiator were collected in this experiment. 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.

Acknowledgements:
The authors gratefully acknowledge the National Research Council of Thailand and Pibulsongkram Rajabhat University for their partial support.

พอลิเมอร์ลอกแบบโมเลกุลสำหรับแยกกรดแกลลิกและกรดเอลลาจิก

Molecularly Imprinted Polymer for Separation of Gallic Acid and Ellagic Acid

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บทคัดย่อ

ได้เตรียมเฟสคงที่พอลิเมอร์ลอกแบบโมเลกุลสำหรับวิเคราะห์ปริมาณกรดแกลลิกและกรดเอลลาจิก เตรียมพอลิเมอร์ด้วยวิธีให้ความร้อนซึ่งประกอบด้วยเบนซอลเปอร์ออกไซด์เป็นตัวเริ่มปฏิกิริยา ตัวทำละลายผสมระหว่างโดเดคานอลและทูลูอีน ไดไวนิวเบนซีนเป็นตัวเชื่อมโยงโมเลกุลและสไตรีนเป็นมอนอเมอร์ ตามลำดับ ทำการวิเคราะห์ปริมาณกรดแกลลิกและกรดเอลลาจิกด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง โดยใช้คอลัมน์ GA-EGA-MIP ขนาด 50×4.6 mm แยกด้วยเฟสเคลื่อนที่ระหว่างน้ำและอะซิโตไนไตรล์ ที่พีเอช 3 (อัตราส่วน 5:95 โดยปริมาตร) อัตราการไหล 0.1 มิลลิลิตรต่อนาที ปริมาตรฉีดสารเท่ากับ 10 ไมโครลิตร และตรวจวัดที่ความยาวคลื่น 280 นาโนเมตร วิธีนี้มีความเหมาะสมสำหรับแยกและทำให้บริสุทธิ์ของกรดแกลลิกและกรดเอลลาจิกจากสารสกัดเปลือกลำไย

คำสำคัญ: พอลิเมอร์ลอกแบบโมเลกุล, โครมาโทรกราฟีของเหลวสมรรถนะสูง, กรดแกลลิก, กรดเอลลาจิก

Abstract

Molecularly imprinted polymers stationary phase was prepared for the determination of gallic acid (GA) and ellagic acid (EGA) by thermal polymerization method using benzoyl peroxide as initiator, the mixtures of dodecanol and toluene as porogens, divinylbenzene as cross-linker and styrene as functional monomer, respectively. Gallic acid and ellagic acid were determined by HPLC using GA-EGA-MIP column, 50×4.6 mm as an analytical 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 injection volume was adjusted to 10 µL, and the absorption was made at 280 nm. This approach method is shown to be successful for the separation and purification of gallic acid and ellagic acid from the extract of *Dimocarpus longan* peels.

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

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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 province. In 2011, the cultivated yield of Longan was approximately 47,580 tons in Chiang Rai and Chiang Mai provinces. For the fruits 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 in a rich variety 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 with 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 many 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). Molecular imprinting technology is a synthetic approach to imitate natural molecular recognition. The printing process is performed by co-polymerizing functional and cross-linking monomers in the presence of a template molecule. The subsequent removal of the imprint molecule reveals binding sites in the polymer network, which are complementary to the template in size and shape. That allows the highly specific rebinding of the template. Furthermore, usually MIPs are 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 to separate and purify of gallic acid and ellagic acid by MIPs and determination of gallic acid and ellagic acid in Longan extract. Moreover, the utilization of fruit processing industry by-products as a material for value-added product preparation would be useful.

2. Materials and Methods

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 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 prepared MIPs was 1:2. The 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 is thermal initiation at 65 °C for 24 h in hot air oven. After thermal initiation, the column is washed with ethanol and acetonitrile using a hand-held syringe to remove unreacted reagents. Non-imprinted polymers (NIPs) were prepared simultaneously under the same conditions without the addition of the template. Microscopic analysis of the monolithic column was performed in Leo1455VP Scanning Electron Microscope.

Apparatus and instruments

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 analytical column used was GA-EGA-MIP (50mm×4.6 mm i.d.). Mobile phase was a mixture containing varying ratios of acetonitrile and milli-Q 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 retention times were determined by injection of mixed standard (10 µg mL^{-1}) of gallic acid and ellagic acid. The triple injections were carried out and the calculated as the final data. Capacity factors (k') were calculated by using the equation $k' = (t_R-t_0)/t_0$, where t_R is the retention time of an analyte and t_0 is the elution time of the void marker. Separation factor (α) was defined as the ratio of the capacity factor of gallic acid and ellagic acid.

Standard solutions

The stock standard solution of gallic acid and ellagic acid were prepared in acetonitrile to provide a concentration 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 hour. 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 hour. 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 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 (blank). Figure 1 shows the scanning electron micrographs for the styrene monoliths, these show a porous surface with pore diameter distributions from 2-5 mm. The effect of backpressure was studied by according to theory for conventional chromatography, 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 length of 50 mm was only 265 kgf at the flow rate 0.1 $mlmin^{-1}$, whereas was only 406 kgf on a long column with length of 100 mm at the same flow rate.

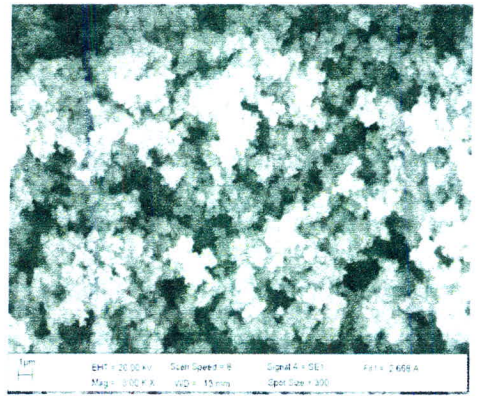

Figure 1: Scanning electron micrographs of the MIPs by thermal-polymerization method

Method Optimization

A precursory experiment was carried to investigate the spectral characteristics of gallic acid. The absorption spectrum was studied by batch wise spectrophotometry, the 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 chromatographic separation was performed at a flow rate of 0.1 mL min^{-1} and using GA-EGA-MIP column, 50×4.6 mm as an analytical 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. Then, the sample solution was separated and determined by HPLC using Ultra C18 column, 5 μm, 150 mm x 4.6 mm (Fig. 2).

Table 1: Intra- and inter day for the studied gallic acid standard solutions (n=7)

Compounds	concentration ($\mu g\ mL^{-1}$)	Intra-day precision % R.S.D	Inter-day precision % 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 2: Analytical recovery of gallic acid and ellagic acid added to Longan sample solution

Longan sample	% Recovery	
	Gallic acid	Ellagic acid
Sample 1	97.01	95.08
	98.12	96.15
	94.08	94.98
Mean±S.D.	96.40±2.08	95.40±.0.64
Sample 2	78.07	88.59
	86.11	86.13
	96.22	96.87
Mean±S.D.	86.79±9.08	90.53±5.62

Table 3: Gallic acid and ellagic acid contents in Longan sample

Sample	Content	
	Gallic acid (μg/g)	Ellagic acid (μg/g)
Sample 1	35.41	9.49
Sample 2	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 $\mu g\ mL^{-1}$ were injected into the HPLC system. The calibration graph was obtained by plotting the absorbance of the solutions against the standard concentrations. Linear calibration graph over the concentration range of gallic acid and ellagic acid were showed 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 standard deviation of the blank (or the intercept of the calibration curve) and S is the slope of 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 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 1.

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 2). Additives and excipients did not interfere in 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 3. The sample was separated using GA-EGA-MIP, 50×4.6 mm. The sample solutions were collected to determination of gallic acid and ellagic acid by reverse phase HPLC method. As shown by HPLC chromatograms of MIP and NIP sample (Fig. 3 and 4), 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 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 functional monomer and 420 μL dodecanol, 180 μL toluene as porogenic solvents, 37 mg BP as initiator were collected in this experiment. 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.

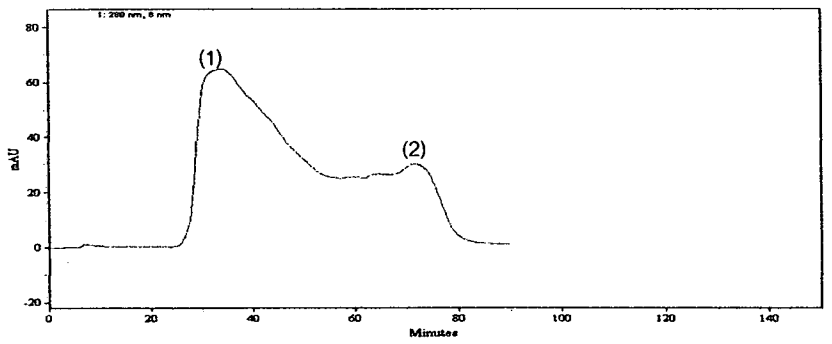

Figure 2: Chromatogram separation of sample using GA-EGA-MIP column, 50×4.6 mm [gallic acid (1) and ellagic acid (2)]

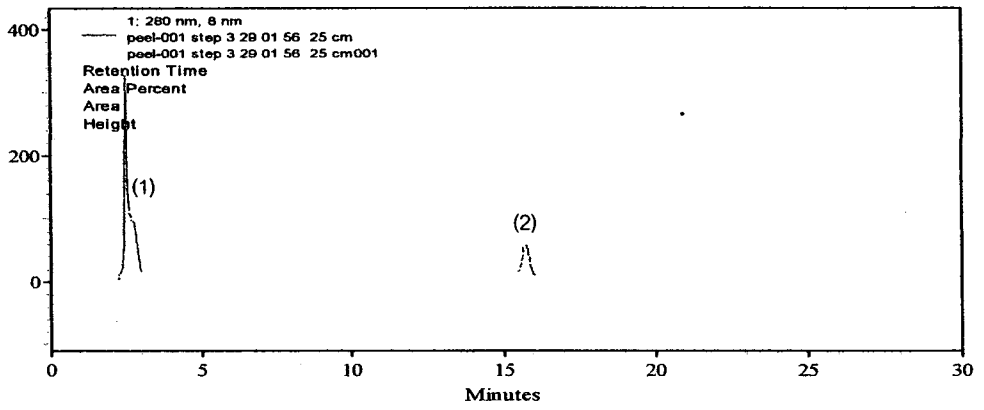

Figure 3: Chromatogram separation of sample using Ultra C18 column [gallic acid (1) and ellagic acid (2)] after load with GA-EGA-MIP

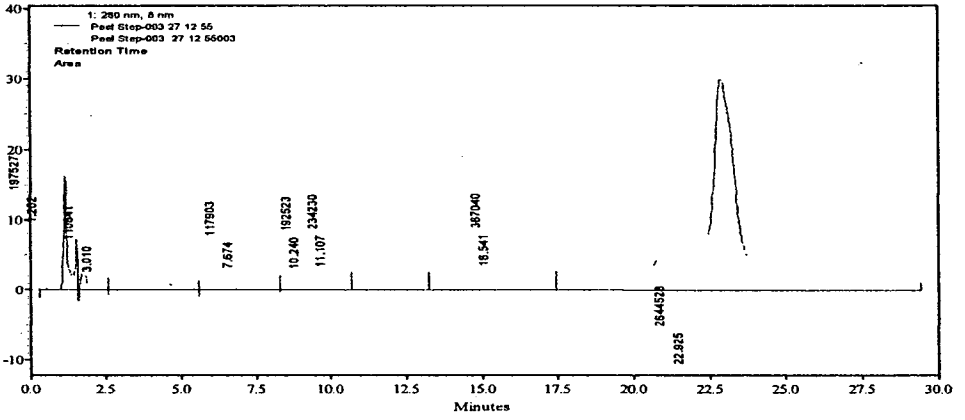

Figure 4: Chromatogram separation of sample using Ultra C18 column, 5 µm, 150 mm x 4.6 mm after load with Non-MIP

Acknowledgements

The authors gratefully acknowledge the National Research Council of Thailand and Pibulsongkram Rajabhat University for their partial support.

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