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Quantitative determination of plant hormones and derivatives in biogas slurry using on-line solid phase extraction with high performance liquid chromatography (On-Line SPE HPLC)

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Abstract. A novel method using on-line solid phase extraction coupled with high performance liquid chromatography (On-line SPE HPLC) was developed to measure three important plant hormones and two indole derivatives in the biogas slurry. These measured substances include abscisic acid (ABA), gibberellins (GA), indoleacetic acid (IAA), tryptophan (TRP) and skatole (IMD), respectively. This method could continuously perform extraction of ABA, GA, IAA, TRP and IMD from different concentrations of biogas slurry samples without complicated pretreatment avoiding lost, and then the extracts were analyzed by high performance liquid chromatography (HPLC). After optimized the analytical condition, a baseline separation of three plant hormones and two indole derivatives was accomplished within 35 mins. The results showed excellent linearity (R2 values of 0.9930-0.9999) and intra- and inter-day precisions (in the range of 3.8-7.2% and 3.9-7.5%), respectively. The limits of detection and quantification were in the range of 0.16-0.47 μg·mL-1 and 0.53-1.57 μg·mL-1, respectively. The determination performance was satisfactory with the proposed method. The results showed that the developed method was suitable for analysis of trace plant hormones in the biogas slurry.

Keywords: biogas slurry; plant hormones; indole derivatives; On-line SPE HPLC

1. Introduction

In recent years, anaerobic digestion has been developed for manure treatment coupled with advantages of bio-energy supply in China. However, the huge amounts of digestate bring pollution risk to the environment [1]. The anaerobic digestate contain not only high concentration of nutrients (Nitrogen, phosphorus and potassium), but also contain plant hormones, bioactive substance and amino acids, etc. [2]. After solid-liquid separation, the solid part named biogas residue usually is used for production of solid organic fertilizer for sale, while the liquid part named biogas slurry is stored in a storage pond. Consequently, there is not enough farmland near the biogas plant for biogas slurry irrigation.

Indeed, biogas slurry is a good kind of high-quality and quick-acting fertilizer. It is very extensively applied in soaking seed [3], topdressing and fertigation [4]. Besides as fertilizer, biogas slurry has other two functions of plant growth regulator and biological pesticide in agricultural production [5]. This can be explained by the presence of plant hormones such as abscisic acid (ABA), gibberellins (GA), indoleacetic acid (IAA) in the biogas slurry [6]. Plant hormones are important natural substances in the plants, playing an important role during the plant's life cycle, such as sprouting, rooting, growth, metabolism and morphogenesis [7, 8]. In addition, the tryptophan (TRP) and the skatole (IMD) were reported as the precursor and derivative of IAA [9]. Therefore, a highly sensitive and comprehensive analytical method of plant hormones will greatly facilitate the investigation of synthesis, metabolic mechanism and dynamics during anaerobic digestion.

Some technical methods have been reported to assay plant hormones in biological matrices, which included enzyme-linked immunosorbent assay (ELISA) [10], GC-MS [11], LC-MS/MS [12-15], and HPLC [16-18]. However, the biogas slurry is a very complex matrix with high concentration of nitrogen, phosphorus, potassium and organic matter. The plant hormones are present at relatively low concentration. For demand for continuous monitoring of a large number of

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samples, these methods above-mentioned are expensive, time-consuming, and sometimes required preconcentration and extraction steps that increase the risk of analyte losses [19]. The comparison of the reported methods for plant hormones is illustrated in Table 1. Compared to off-line SPE, the sample pretreatment time and cost are significantly reduced by using on-line SPE pretreatment [20-25]. Meanwhile, pretreatment procedures and sample analysis procedures of two samples can be staggered by equipping with automatic sampler. Furthermore, the analyte losses and sample contamination can be effectively avoided by using on-line SPE.

Table 1. Comparation of different methods for assay plant hormones

Method	Pretreatment	Time spent	characteristic	Reference
ELISA	Dissolved, extracted, evaporation in vacuum, mixed with monoclonal antibody	More than 12 hours	high sensitivity, laborious, inevitable cross-reactivity of the antibodies, poor specificity, accuracy, or reproducibility	[10]
GC–MS/MS	Frozen, dissolved, centrifugation, ultrasonic treatment, microscale solid-phase extraction, methylation	4-5 hours	high sensitivity, complicated derivatization steps, the high temperature in the GC injector would lead to the thermal breakdown of analytes.	[11]
LC-MS	Frozen, extracted for at least 16h, centrifugation, re-extracted, solid phase extraction, evaporated	17 hours	good sensitivity and accuracy, high cost of equipment, the requirement of skillful operator	[12-15]
Off-line HPLC	Dissolved and extraction for many times, evaporated under a stream of nitrogen, protein precipitation	4-5 hours	Laborious, time consuming, low recovery	[16-18]
On-line HPLC	Centrifugation, extracted automatically	1 hour	reduces the sample preparation time, decreases the analyte losses and sample contamination	[20-25]

In this study, an on-line SPE HPLC system with automated high throughput was developed to determine plant hormones in biogas slurry. At the beginning, the sample was firstly loaded into the on-line SPE column (Acclaim C18), on which the target analytes can be retained, while the impurity substances were washed away. Subsequently, the analytes were automatically transferred

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controlled by six-port injector valve from SPE column to the analytical HPLC column for analysis. After optimizing the conditions, the method can be used for accurate, quick, and cost-effective analysis of large numbers of biogas slurry samples. Overall, this work was aimed at promoting a better elaboration of production and metabolism pathway of plant hormones in biogas slurry which was important for valorization of biogas slurry.

2. Materials and methods

2.1 Reagents and materials

The standards: TRP, GA, IAA, ABA, IMD were obtained from Sigma-Aldrich Laboratories, Inc. (St. Louis, MO, USA). All analytes were dissolved in HPLC grade methanol which was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical reagent grade. Ultra-pure water was obtained from a Milli-Q water system (Millipore, Billerica, MA, USA).

2.2 Instrumentation and methods

The analysis was performed using a Dionex Ultimate U3000 system (Dionex, Sunnyvale, USA), which equipped with an on-line SPE column, an analytical column, a six-port switching valve integrated in a column oven, dual-gradient pump (loading pump and analytical pump), an autosampler with a 100 μL sample loop, and a diode array detector (DAD). The on-line system is shown in Figure 1. The SPE column is a part of the sample preparation system, and the analytical column is a part of the separation system.

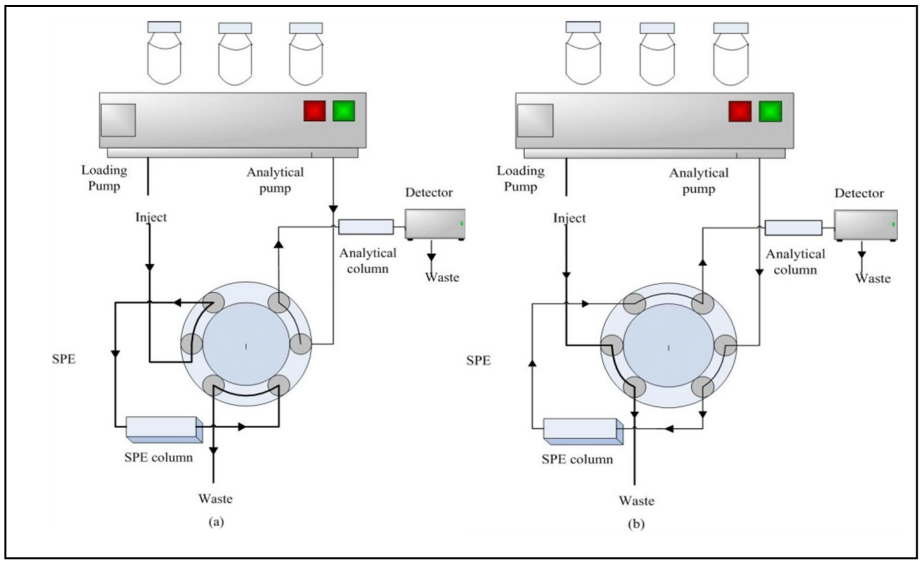

Figure 1. Configuration of on-line SPE HPLC-DAD system: (a) step 1 and step 3 were in valve position 1; (b) step 2 was in valve position 2.

A PA II C18 guard column (10 mm × 4.6 mm, 5 μm) was chosen as the on-line SPE column for sample pretreatment, while an Acclaim® Polar Advantage II (PA II) C18 (150 mm × 4.6 mm, 5 μm) was used as analytical column for separation the target analytes. All system control, data acquisition and analysis were performed with the Chroméléon software (Dionex, Sunnyvale, USA). The mixture of methanol and water was used as mobile phase which was filtered by a millipore device with microfiber filters (4.5 μm; Phenomenon, Tianjin, China).

The analysis was carried out in three steps after the biogas slurry sample (50 μL) was injected into the SPE column. Firstly, the sample was washed with 5% MeOH:95% H2O in SPE column by the loading pump at a flow rate of 0.8 mL·min-1 to flush endogenous matrix into the waste for 6 min, meanwhile analytes were extracted on the SPE column (Fig. 1a). Secondly the analytes were back eluted from SPE column into the analytical column by analytical pump (Fig. 1b). The last step

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was the separation of analytes by gradient elution with methanol-water in the analytical column by the analytical pump (Fig. 1a).

The recovery of the analyte was employed to evaluate the adsorption efficiency. The recovery was calculated by the following equation:

$$R_c = \frac{C_2 - C_1}{C_0} \quad (1)$$

Where RC is the recovery of the analyte, C0 is the concentration of spiked analyte, C1 is the concentration in extracted unspiked sample and C2 is the concentration in extracted spiked sample, respectively.

2.3 Preparation of standards and quality control materials

The initial stock solution was prepared by dissolving each analyte in HPLC grade methanol at a concentration of 10 mg·mL-1, which was stored at -20 ℃. The working solutions were made by diluting the stock solution serially with ultra-pure water. Calibration samples at the concentrations of 1, 3, 5, 7, 10, 30, 50, 70, and 100 μg·mL-1 were prepared. All these solutions were stored at 4 ℃.

The calibration curve, which consisted of nine calibration concentrations (each concentration was analyzed in five replicates), was constructed by plotting the peak area versus each analyte concentration.

2.4 Sample preparation

Biogas slurry was obtained from three kinds of anaerobic digestion reactors which were feed different materials including chicken manure, cattle manure and pig manure. Biogas slurry was a kind of complex matrix. To maximize the lifetime of the SPE and HPLC analytical columns, the pH of the biogas slurry was adjusted to 2.5 by adding formic acid to prevent the ionization of analytes and centrifuged for 20 min at 8000 rpm. Finally, the supernatant was percolated through 0.22 μm nylon filter to remove suspended matters and then transferred to sample vials. All assays were performed in three duplicates.

3. Results

3.1 Optimization of on-line SPE-HPLC analytical conditions

Because the biogas slurry contains high concentration of impurity substances, the Acclaim® Polar Advantage II (PA II) C18 was chosen as the pre-column for cleaning the sample and enrichment of the analytes. The C18 material possesses long polarity sulfanilamide chains groups on the surface of 120 Å silica. The structure can keep small molecules and limit the access of large molecules substances. Besides the column, the condition of sample loading flow rate, adsorption time and the separation condition of analytical column was investigated systematically.

3.1.1. Effect of loading flow rate on the recovery

The loading flow rate affected the pre-concentration adsorption efficiency of the analytes during the clean-up step. Low flow rate was attributed to increase more contact time between the analytes and the sorbent surface to improve pre-concentration efficiency. Otherwise, high flow rate could shorten the adsorption time and improve the sample throughput. Figure 2 shows the recoveries of 50 μL of spiked (50 μg • mL-1) purified water samples. The samples were pre-concentrated in SPE column at different rates ranged from 0.5 to 1.5 mL • min−1. The recoveries of the analytes decreased with increasing of the sample loading flow rate. The decrease ranges of sample recovery are little at the flow rate of 0.5 to 0.8 mL • min−1, but it decreases quickly when the flow rate is greater than 0.8 mL • min−1. Therefore, 0.8 mL • min−1 was adopted as the optimal sample loading flow rate in this study. As the results shown in Fig. 2, the recoveries of five analytes were greater

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than 94% at the flow rate of 0.8 mL • min−1. It showed that the SPE column could concentrate and extract analytes effectively.

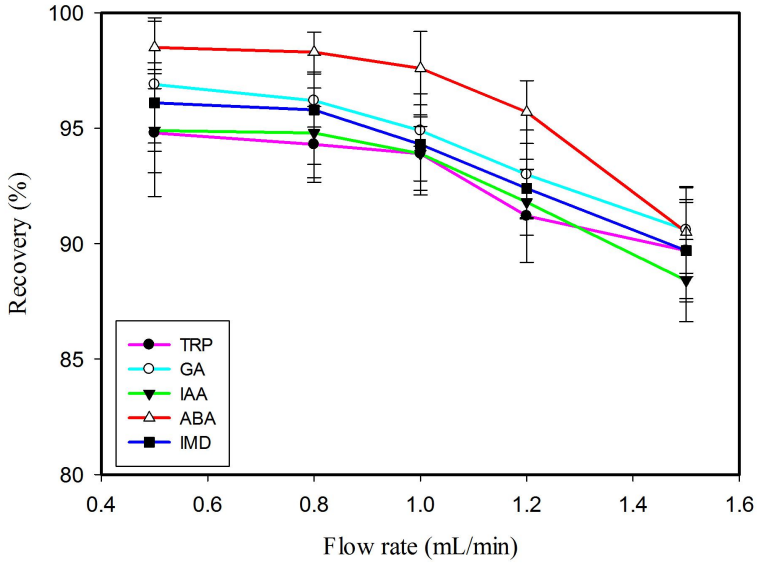

Figure 2. The recoveries of the analytes at different sample loading flow rate

3.1.2. Effect of elution time on the recovery

When the pretreatment step was completed, the elution of the analytes from the SPE column to the analytical column was a critical step. Too short elution time will cause a poor recovery, while long elution time will make many matrix substances be transferred to the analytical column. The elution time was optimized by using standard solution. As shown in Figure 3, the recoveries of the plant hormones increased remarkably as the elution time from 2 to 4 min. When the elution time was longer than 4 min, the recoveries of plant hormones keep equation and decreased slightly after 5 min. Therefore, optimizing elution time of 4 min was selected accordingly and the six-port injector valve was turned to the “load” position for next pre-concentration.

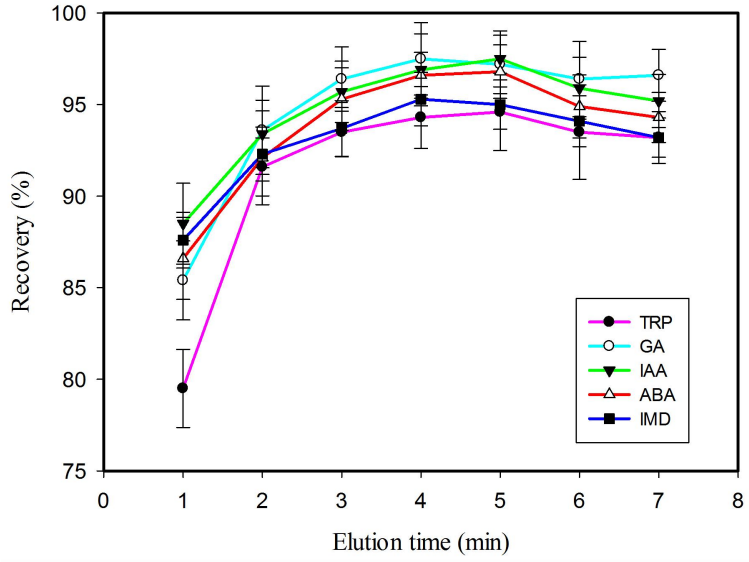

Figure 3. The recoveries of the analytes under the different elution time

3.1.3 Optimization of the separation condition of analytical column

The separation condition of analytical column was the most important parameter to obtain an optimized procedure. To simplify the method, it is better to introduce the same organic solvents as

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the SPE column. Thus, the methanol and water were chosen as the mobile phase of analytical column. Under the condition, the pretreatment and HPLC separation were carried on at the same time without introducing other organic solvents into the system.

For this purpose, the program was optimized to ensure baseline separation of the five analytes synchronously. Various ratios of methanol to water were tested as the mobile phase. The higher ratio of methanol to water resulted in incomplete baseline separation of the ABA, IAA and IMD. Oppositely, the lower ration of methanol to water caused longer separation time and wide chromatographic peaks. After exploration, we got the gradient program used for both separation and desorption (shown in Table 2). The ratios of A to B and C to D are the mobile phase for the loading pump and analytical pump, respectively. The experiment was implemented under this condition.

Table 2. An overview of the on-line SPE HPLC method

Time (min)	Loading Pump: A—methanol B—2% acetic acid solution			Time (min)	Analytical Pump: C—H_2O D—methanol			Six-valve position
	Flow rate (mL·min^{-1})	A (%)	B (%)		Flow rate (mL·min^{-1})	C (%)	D (%)	
0	0.8	30	70	0	0.5	95	5	1-6
6	0.8	30	70	6	0.5	95	5	1-2
25	0.8	70	30	10	0.5	70	30	1-6
26	0.8	100	0	13	0.5	5	95	
30	0.8	100	0	17	0.5	5	95	
32	0.8	30	70	20	0.5	70	30	
35	0.8	30	70	28	0.5	95	5	
				35	0.5	95	5	

Fig. 4 shows the HPLC chromatogram of the five analytes. The resolution of two neighboring peaks was employed to evaluate the optimum separation condition. The resolution was evaluated using the following equation:

$$R_s = \frac{2(t_{R2}-t_{R1})}{w_2+w_1} \quad (2)$$

Where Rs is the resolution of two neighboring peaks, tR1 and tR2 are the retention times for the first and second peaks, w1 and w2 are the peak widths of the two neighboring peaks, respectively. When Rs is equal to 1, the peaks are 98% resolved. The resolutions of TRP-GA, GA-IAA, IAA-ABA, ABA-IMD shown in the Figure 4 are 6.25, 8.09, 4.39, 11.22, respectively. It implied that adequate separation was achieved.

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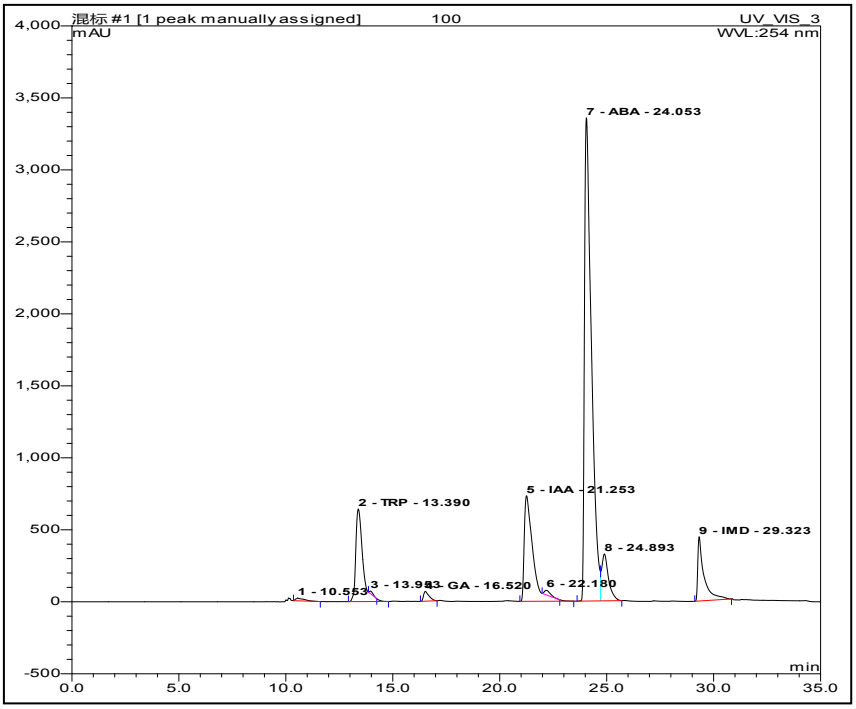

Figure 4. On-line SPE-HPLC chromatograms of 50 μL standard solution spiked at 50 μg • mL-1

with each analyte compound. 2- tryptophan (TRP), 4- gibberellins (GA), 5- indoleacetic acid (IAA), 7- abscisic acid (ABA), 9- skatole (IMD).

3.2 Validation of the method

Various concentrations ranging from 0.5 to 100 μg • mL-1 of these plant hormones were analyzed to test the calibration curve (shown in Table 3). Calibration curves were obtained for each compound by five-point calibration with correlation coefficients generally greater than 0.993.

Table 3. The analytical characteristic data of the developed on-line solid-phase extraction coupled with HPLC for the determination of five analytes

Analyte	retention time (min)	Linear equation	Correlation coefficient(R^2)	linearity range ($\mu g \cdot mL^{-1}$)	LODs ($\mu g \cdot mL^{-1}$)	LOQs ($\mu g \cdot mL^{-1}$)
TRP	13.380	Y=0.6309X+0.0102	0.9982	0. 50-50.00	0.24	0.80
GA	18.043	Y=4.873X+0.0332	0.9930	1.00-100.00	0.16	0.53
IAA	21.253	Y=1.3610X+0.0178	0.9999	1.00-100.00	0.45	1.50
ABA	24.080	Y=2.0211X+0.0326	0.9998	1.00-100.00	0.47	1.57
IMD	29.500	Y=0.8552X+0.0439	0.9980	1.00-100.00	0.39	1.30

The minimum concentrations of identifying and quantifying the analyte by the method were defined as Limit of detection (LOD) and limit of quantification (LOQ), respectively, calculated by signal/noise ratio of 3:1 and 10:1. The LODs and LOQs of the plant hormones were in the range of 0.16-0.45 μg • mL-1 and 0.53-1.57 μg • mL-1, respectively (Table 3). Vishal Gupta (2011) reported that the LODs of ABA and IAA were 0.5 μg •mL-1 and 1 μg •mL-1, respectively, using dispersive liquid-liquid microextracton method[17]. The other extraction method based on solid-phase micro-extraction (SPME) also showed LODs as lower as 0.2 μg • mL-1 for IAA[26]. Panadda tansupo (2010) obtained the LODs of 0.1 μg •mL-1 for IAA, 3.5 μg •mL-1 for GA, 2.0

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μg • mL-1 for ABA[27]. The LODs obtained using the method developed in our study were lower or equal to other reported studies [16, 28, 29].

The intra- and inter-day relative standard deviations (R.S.D.s) were measured to evaluate the precision of the method. The spiked samples at three different concentration (5, 50 and 100 μg • mL-1) were analyzed six times in one day to calculate the intra-day precision and over 3 days to calculate the inter-day precision. As shown in Table 4, the R.S.D.s of intra- and inter-day are from 3.8% to 7.2% and from 3.9% to 7.5%, respectively.

Table 4. The intra- and inter-day precisions and recoveries of the assay (n=6)

Analyte	Intra-day precision						Inter-day precision					
	5 μg·mL^{-1}		50 μg·mL^{-1}		100 μg·mL^{-1}		5 μg·mL^{-1}		50 μg·mL^{-1}		100 μg·mL^{-1}	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
TRP	95.4	3.8	93.5	4.8	98.5	5.2	93.6	7.2	91.6	6.7	94.5	6.9
GA	96.7	5.2	96.7	5.7	98.4	3.9	97.7	7.2	92.4	7.5	95.1	7.2
IAA	93.4	6.8	95.6	6.9	96.3	4.8	94.6	5.7	98.3	3.9	92.7	5.4
ABA	92.6	7.2	94.8	5.4	95.2	4.6	93.8	4.9	95.5	4.6	94.4	5.7
IMD	97.7	4.8	96.2	5.2	92.5	6.6	96.2	5.5	95.9	4.5	93.6	6.3

+ R.S.D-Relative standard deviation

The recoveries of five analytes were measured and compared by the determination of the analytes in 1.0 mL high purity water and in pig manure biogas slurry spiked at 5, 50 and 100 μg • mL-1 levels. The pig manure biogas slurry sample was selected as reference matrix. The recoveries of five analytes are the range of 92.6-98.3% in the high purity water and 91.3-98.2% in the biogas slurry, respectively. Recoveries of five analytes in biogas slurry were not different statistically with those in high purity water (Table 5). That is, the test results were not affected by matrix effects.

Table 5. Recoveries of five analytes in high purity water and pig manure biogas slurry samples (% mean±SD, n=3)

Analyte	High purity water			Pig manure biogas slurry		
	5 μg·mL^{-1}	50 μg·mL^{-1}	100 μg·mL^{-1}	5 μg·mL^{-1}	50 μg·mL^{-1}	100 μg·mL^{-1}
TRP	92.8±7.4	94.3±5.4	94.3±7.4	94.9±9.2	91.3±9.2	93.7±7.8
GA	96.4±8.1	96.2±9.3	97.5±8.9	96.8±6.1	94.6±9.9	95.2±6.6
IAA	92.6±4.5	94.8±6.7	96.9±8.8	94.3±5.9	97.0±8.0	96.1±5.1
ABA	95.3±5.2	98.3±8.9	96.6±5.9	92.6±8.8	90.4±4.9	98.2±5.4
IMD	98.2±4.6	95.8±9.9	95.3±9.8	95.6±8.7	96.5±5.8	94.8±6.7

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3.3 Biogas slurry sample analysis

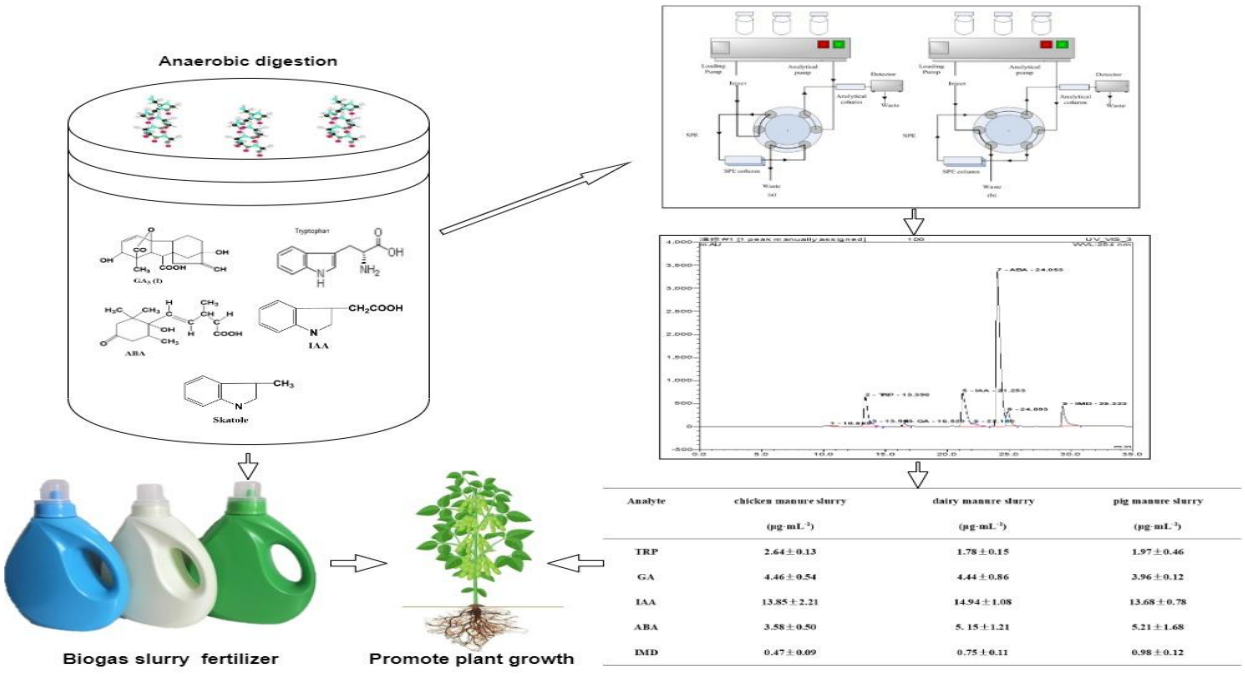
Table 6 demonstrates the testing results of three types of biogas slurry samples by using the developed method. All analytes were detected in three types of biogas slurry. The concentration of IAA in this study was close to the concentration (33.0 nmol • g-1) reported by D.Kostenberg[30]. D.Kostenberg reported the biosynthesis of IAA during anaerobic digestion of instant coffee waste. The results showed that the method developed in this study is practical in analysing the biogas slurry.

Table 6. The analytes concentration of the biogas slurry

Analyte	chicken manure slurry ($\mu g \cdot mL^{-1}$)	dairy manure slurry ($\mu g \cdot mL^{-1}$)	pig manure slurry ($\mu g \cdot mL^{-1}$)
TRP	2.64±0.13	1.78±0.15	1.97±0.46
GA	4.46±0.54	4.44±0.86	3.96±0.12
IAA	13.85±2.21	14.94±1.08	13.68±0.78
ABA	3.58±0.50	5. 15±1.21	5.21±1.68
IMD	0.47±0.09	0.75±0.11	0.98±0.12

4. Conclusion

A sensitive, selective and precise automated on-line SPE-HPLC method was developed to measure simultaneously three plant hormones (ABA, GA, IAA) and two indole derivatives (TRP, IMD) in biogas slurry. The LODs and LOQs of this method, in the range of 0.16-0.45 μ g • mL-1 and 0.53-1.57 μ g • mL-1, are lower or equal to other reported studies. The R.S.D.s of intra- (3.8% -7.2%)and inter-day (3.9% -7.5%) showed the method was precise for biogas slurry analysis . The effectiveness of this method was evaluated by the successful identification and quantification of ABA, GA, IAA, TRP, IMD in three anaerobic digested slurries. The developed method was more simple, sensitive and precision for the screening and analysis of plant hormones in anaerobic biogas slurry. The method did not need complicated and time-consuming sample pretreatment and could be used for quick, accurate, and cost-effective analysis of large numbers of samples. So it has a practical significance for screening of plant hormones in anaerobic biogas slurry.



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