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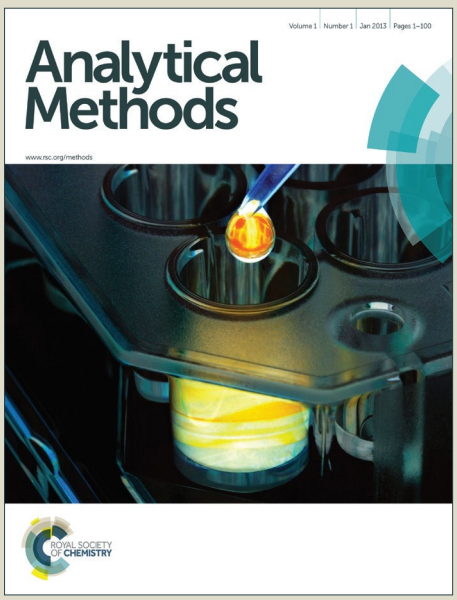

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Development of microwave-assisted extraction and liquid chromatography-tandem mass spectrometry for determination of maleic hydrazide residues in tobacco

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Herein, an effective and rapid method involving microwave-assisted extraction (MAE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed for the determination of maleic hydrazide residues in tobacco. In order to completely extract the free and bound maleic hydrazide, MAE was employed to accelerate the hydrolysis of the maleic hydrazide glycoside, which was proved to be a powerful extraction method in comparison with conventional extraction methods. Then the obtained extract was filtered and directly analyzed by LC-MS/MS without further pretreatment, and d_2- maleic hydrazide was used as the internal standard to reduce the matrix effects. A dynamic range of 50~5000 ng/mL was achieved with limit of detection of 0.16 mg/kg for maleic hydrazide. The established method exhibited good repeatability and recovery for maleic hydrazide, and could be used as a rapid and reliable approach for routine analysis of maleic hydrazide in tobacco.

Introduction

Maleic hydrazide (MH) is one of the most effective plant growth regulators, which has been extensively used in many countries. It is reported that most of the MH used in the United States is applied to tobacco cultivation.[1] Although MH has not been proved to be related to cancer disease, it has been shown to cause genotoxic effects in some mutagenicity studies and remains great uncertainty about its chronic toxicity to non-target organisms.[2-4] As the MH residues in tobacco could be transferred to the cigarette smoke with relatively high transfer rate, the Cooperation Center for Scientific Research Relative to Tobacco (CORESTA) guidance residue level (GRL) established for MH is currently set at 80.00 mg/kg.[5]

Up to now many different methods have been established for the extraction of MH from a range of commodities, including potato,[6-9] garlic,[10] mixed vegetal matrices.[11,12] However, it has been confirmed that the MH in tobacco plants would participate in the formation of β-d-glucoside,[13,14] thus the glucoside conjugates will not contribute to the MH result unless it is hydrolyzed prior to analysis. The earlier colorimetric approach recommended by CORESTA have been widely used in the tobacco industry. However, such procedures have a low sample capacity; they are not specific and susceptible to interferences. Furthermore, the application of these procedures presents a safety issue due to the use of hot alkali. [15] Heat reflux extraction and ultrasonic extraction have been reported to extract both the free and bound MH in tobacco simultaneously,[16,17] but these protocols are laborious, time-consuming and require large volumes of toxic solvents. Microwave-assisted extraction (MAE) has been accepted as a potential and powerful technique for the extraction of organic compounds from plant samples. It possesses many advantages, such as saving in processing time and solvent consumption, and has been widely used in many areas.[18-22] In this work, MAE is used for the extraction of both free and bound MH in tobacco.

Different analytical techniques have been applied for the determination of MH residues, such as GC, HPLC, LC-MS/MS and CE.[23-26] And only GC and HPLC have been applied for the analysis of MH residues in tobacco. As MH is nonvolatile, prior derivatization is required before GC analysis.[16] While for HPLC, MH could be directly analyzed. According to the reported articles,[17,24] reversed-phase liquid chromatography (RPLC) has been widely used for the MH residues analysis. However, due to the highly polar of MH, RPLC utilizing C8 or C18 columns could not provide a good separation of the principal components of tobacco sample as a result of the complex matrix. Pan et al. has employed the ion-exclusion chromatography for MH residues determination in vegetables.[25] Lewis et al. reported the coupling of strong anion exchange chromatography and tandem mass spectrometry for determination of MH residues in potato chips.[7] Instead of using internal standard, the matrix spiked with MH standard was used to build the calibration curve to reduce the matrix effects. Although these methods provide a good separation for the complex matrix, complicated clean-up procedures are required to reduce the interference. Thus it is necessary to establish a

sensitive and feasible method for the determination of MH residues in tobacco.

In this study, a rapid method is proposed for the determination of MH residues in tobacco. MAE was employed to simplify the pretreatment procedure and the MAE conditions were evaluated. The obtained extract was directly analyzed by LC-MS/MS without clean-up procedure. To ensure accurate quantitative results, d_2-MH is used as the internal standard (IS). Finally, the developed method was applied for the analysis of MH in real samples.

Material and methods

Materials and chemicals

The MH and d_2-MH of ⩾99.8% purity were purchased from AccuStandard Inc. (New Haven, CT, USA). The hydrochloric acid (HCl), acetic acid and acetonitrile were of HPLC grade from Merck Millipore (Billerica, MA, USA). All aqueous solutions were prepared using deionized water by Milli-Q system (Millipore, Bedford, MA, USA). The tobacco leaves were supplied by Shanghai Tobacco Co. Ltd.

Preparation of standard solutions

Standard stock solutions containing 100 μg/mL MH and the IS stock solution containing 100 μg/mL d_2-MH were prepared in deionized water respectively. The working solutions at various concentrations (5000, 2000, 1000, 500, 200, 100 and 50 ng/mL respectively) containing 1 μg/mL IS were obtained by diluting the stock solutions in deionized water. All the pesticide solutions were stored at 4 ºC in the refrigerator.

Sample preparation

Tobacco leaves were analyzed in this work. After dried at 40 ºC in a hot air cabinet with ventilation for 5 h, samples were triturated, passed through a 0.45-mm stainless steel sieve, and stored in closed desiccators.

Sample pretreatment

MAE

MAE was performed on a MARS5 from CEM Corporation (Matthews, NC, USA). About 0.5 g of the tobacco samples were weighed into the 100 mL extraction vessels. 20mL 2M HCl aqueous solution and 200 μL of IS stock solution were added in each vessel. The vessels were closed gastight, and were then shaken vigorously by hand for 10 s. Sets of 12 vessels were microwave-digested according to the following operational program. The microwave power is 1200 W, the temperature ramps to 180 ºC during 10 min, and then it is held at 180 ºC for 30 min and finally naturally cooled to room temperature. The supernatant was filtered with 0.22 μm PTFE syringe filter (Agilent Technologies, USA) for the LC-MS/MS analysis.

Heat reflux extraction

0.5 g of tobacco samples were introduced into a 250 ml flask and extracted by 40ml of 2M HCl aqueous solution with 400 μL of IS stock solution added for 1 h under reflux. After cooling and filtration, the obtained solution was collected for the LC-MS/MS analysis.

Ultrasonic extraction

0.5 g of the tobacco samples was placed in a 50 mL glass vial. After adding 20mL of 2M HCl aqueous solution and 200 μL of IS stock solution, the vial was capped and placed in an ultrasonic bath containing hot water at a temperature of 80 ºC and sonicated for 1 h. After cooling and filtration, the obtained solution was collected for the LC-MS/MS analysis.

Liquid-solid extraction

0.5 g of the tobacco samples was placed in a 50 mL glass vial. After adding 20mL of extraction solution (0.2M HCl aqueous solution/methanol, 80/20, v/v) and 200 μL of IS stock solution, the vial was capped and shaken for 1 h. After filtration, the obtained solution was collected for the LC-MS/MS analysis.

LC-MS/MS analysis

The LC-MS/MS analysis was performed on an Agilent 1200 HPLC system coupled with an Agilent G6410B triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) interface. The LC separation was performed at 20 ºC on a hypercarb column (5μm, 100 mm × 4.6 mm i.d.) from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The mobile phases consisted of 1% acetic acid aqueous solution (phase A) and acenitrile with 1% acetic acid (phase B). The flow rate was maintained at 0.3 mL/min, and the following gradient elution program was used: 0 min, 100% A; 8 min, 85% A; 15 min, 85% A; 16 min, 10% A; 20 min, 10% A. Finally, the initial conditions were held for 15 min. The sample injection volume was 10 μL. The LC mobile phase flow was connected to MS detector at 8min using a switching valve, and then switched to waste line at 15min.The mass spectrometer was operated under the following parameters for positive ESI: capillary voltage 5000 V, quadrupole temperature 100 ºC, drying gas temperature 350 ºC, drying gas flow rate 10 L/min, and nebulizer gas pressure 60 psi. The quantification measurements were performed in multiple reaction monitoring (MRM) mode. The optimal settings for compound-dependent MS/MS are shown in Table 1.

Results and discussion

Optimization of LC-MS/MS analysis

In this work, a hyprcarb column was used for the MH analysis because of its good retain for polar compounds. And d_2-MH was used as the IS to reduce the matrix effects. As demonstrated in Fig. 1, the retention time of MH is 10.4 min, and the developed method provided a good separation for MH analysis without any clean-up procedure. Compared with the reported work, the developed method is simpler and more reliable.

Table 1. MS/MS parameters for MH and d_2-MH

Analyte	Type	MRM transition (M/Z)	Fragmentor (V)	CE[a] (eV)	Dwell (ms)	Quantifier[b]/Qualifier ion ratios (%)
MH	Quantifier	113→85	100	20	200	189.7

	Qualifier	113→67	100	20	200	
d_2-MH	Quantifier	115→87	100	20	200	287.3
	Qualifier	115→69	100	20	200	

a) Collision energy
b) The value for quantifier ion is 100%

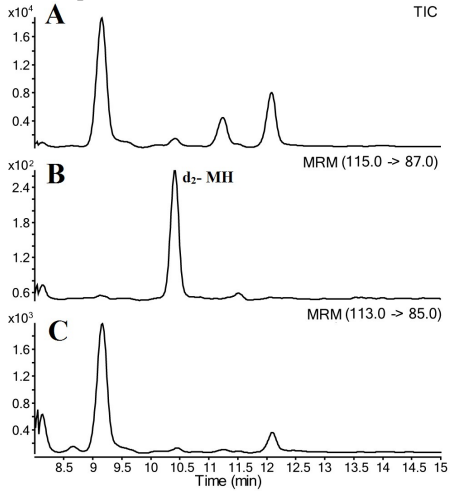

Fig. 1 Chromatograms obtained from blank tobacco sample spiked with the d_2-MH. (A) Total ion chromatogram. (B) extracted ion chromatogram (m/z 115→87). (C) extracted ion chromatogram (m/z 113→85).

Optimization of sample pretreatment

Study of different extraction methods

Two different tobacco samples were employed to study the extraction efficiency of four different methods including MAE, heat reflux extraction, ultrasonic extraction and liquid-solid extraction. Among which, heat reflux extraction and ultrasonic extraction are the two main methods which have been reported to hydrolyze the bound or conjugate form of MH residues. As demonstrated in Fig. 2, the amount of MH extracted by ultrasonic extraction or liquid-solid extraction is lower than MAE and heat reflux extraction, which may be caused by the lower extraction temperature. Comparing MAE with heat reflux extraction, no obvious difference in extraction efficiency was observed. Considering that MAE needs shorter extraction time and lower solvent consumption, MAE was used to extract the MH in tobacco in this work.

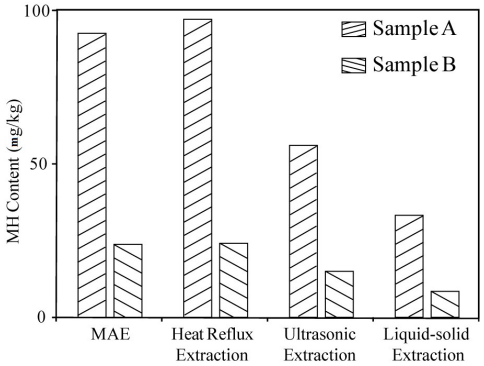

Fig. 2 Study of extraction efficiencies of different sample pretreatment methods.

Optimization of MAE conditions

In order to evaluate the effects of the extraction temperature, MAE was performed at 100 ºC, 120 ºC, 140 ºC, 160 ºC, 180 ºC and 200 ºC respectively for 1 h. Meanwhile, the recovery of the established method was investigated to evaluate the extraction efficiency by spiking 2 μg, 10 μg, 20 μg MH into 0.5 g tobacco sample respectively. As shown in Fig. 3A, the amount of the extracted MH raised as the extraction temperature increased. However, no obvious increase of the extracted MH amount was found, after the extraction temperature increased to 180 ºC or above. And the recoveries of all the different conditions were between 94.3% and 112.8%, which was in line with the accepted quality requirements of the Working Party on Pesticide Residues, recoveries being deemed acceptable if between 80 and 110%. Then 180 ºC was identified as the optimal extraction temperature.

The concentration of HCL aqueous solution were also optimized by using 0 M, 1 M, 2 M, 3 M, 4 M and 5 M HCl aqueous solution at 180 ºC for 1 h respectively. According to Fig. 3B, no more increasement of the extracted MH amount was found when the concentration of HCl reached to 2 M or above. Meanwhile, the recoveries with 0 M or 1 M HCl aqueous solution were found to be lower than 90%. But when the concentration of HCl reached to 2 M or above, the recoveries were between 97.6% and 108.9%. Thus 2 M HCl aqueous solution was chosen for MH extraction.

Finally, the MAE was conducted for 0.5 h, 1 h, 1.5 h and 2 h respectively, to study the effects of the extraction time. As demonstrated in Fig. 3C, all the recoveries under different conditions were satisfied for MH analysis, and 0.5 h was enough to fully extract the MH in tobacco. Thus 0.5 h was fixed as the extraction time regarding the time efficiency.

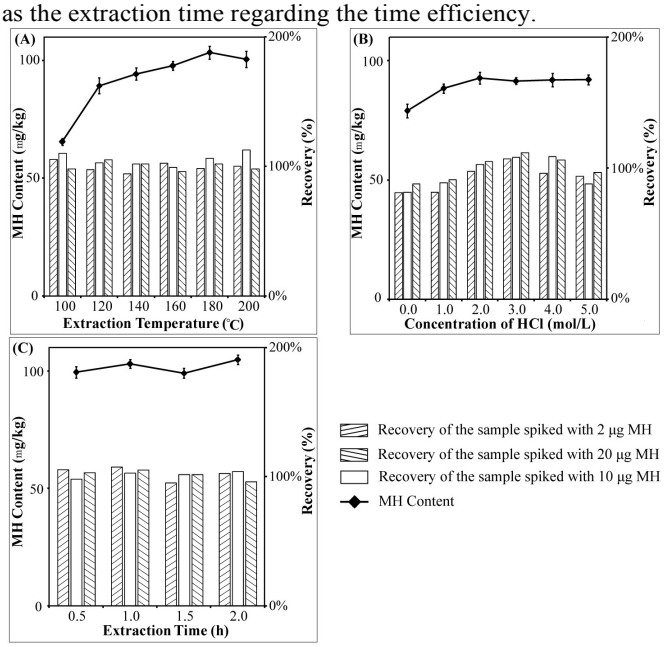

Fig. 3 Study of the MAE conditions.

Method validation

The developed method showed a wide linear range (from 50 to 5000 ng/mL) with determination coefficients (R^2) greater than 0.9996. The recoveries were determined by spiking 2μg, 10μg, 20μg MH into 0.5 g tobacco samples respectively, and all the

recoveries were between 89.6% and 123.5%, which ensured the accuracy of the amount of MH detected in tobacco samples. To evaluate the precision of the proposed method, analysis of tobacco samples spiked with standard solutions of MH were performed under the optimized procedure (n=9). RSDs were found to be 3.3~6.6%. In order to estimate the limit of detection (LOD) and quantification (LOQ) of the proposed method, MH standard was spiked into blank tobacco samples. LOD and LOQ were calculated using signal to noise ratios of three and ten, respectively. The LOD for MH was 0.16 mg/kg, and the LOQ for MH was found to be 0.53 mg/kg. The established method is sensitive enough for routine analysis of MH residues in tobacco samples.

The comparisons of the developed method with the other reported method, including GC-MS[16] and HPLC[17], were summarized in the Table S1. The proposed method presents two main advantages for routine analysis. With the application of MAE and hypercarb column, the pretreatment procedures were simplified, and complicated clean-up procedures were avoided. Meanwhile, due to the high sensitivity of LC-MS/MS, the developed method provides good linearity, precision, reproducibility and low LOD.

Analysis of real samples

Ten tobacco samples (including six imported tobacco samples and four domestic tobacco samples) were analyzed under the proposed method and the results were presented in Table 2. MH is identified by the retention time and ion pair ratio, and the difference between calibration standards and samples should be within 20%. The ion pair ratios of the two transitions in tobacco samples and in the calibration standard are showed in the Table S2. As the result demonstrated, MH residues were detected in five imported tobacco samples while the contents were all under the GRLs regulated by CORESTA. No MH residues were found in domestic tobacco samples.

Table 2. Contents of MH detected in tobacco samples by MAE-LC-MS/MS

Samples	MH Content (mg/kg)
Imported Sample A	62.4
Imported Sample B	35.2
Imported Sample C	30.0
Imported Sample D	54.5
Imported Sample E	53.5
Imported Sample F	n.d.[a]
Domestic Sample G	n.d.
Domestic Sample H	n.d.
Domestic Sample I	n.d.
Domestic Sample J	n.d.

a) Not detected.

Conclusions

In this work, a novel method for the determination of MH residues in tobacco is proposed. It is the first time that MAE is used to fully extract the free and bound MH residues in tobacco samples. In comparison with other reported sample pretreatment methods, MAE provides high extraction efficiency, and is much faster and simpler, which provides an alternative way for MH extraction in complex matrix. Meanwhile, the usage of hypercarb column supported a good separation for MH and simplified the sample pretreatment. In addition, the d_2- MH has been used as the IS to eliminate the matrix effects. All the results demonstrate that the developed method is simple, time and solvents saving, highly sensitive for MH residues analysis.

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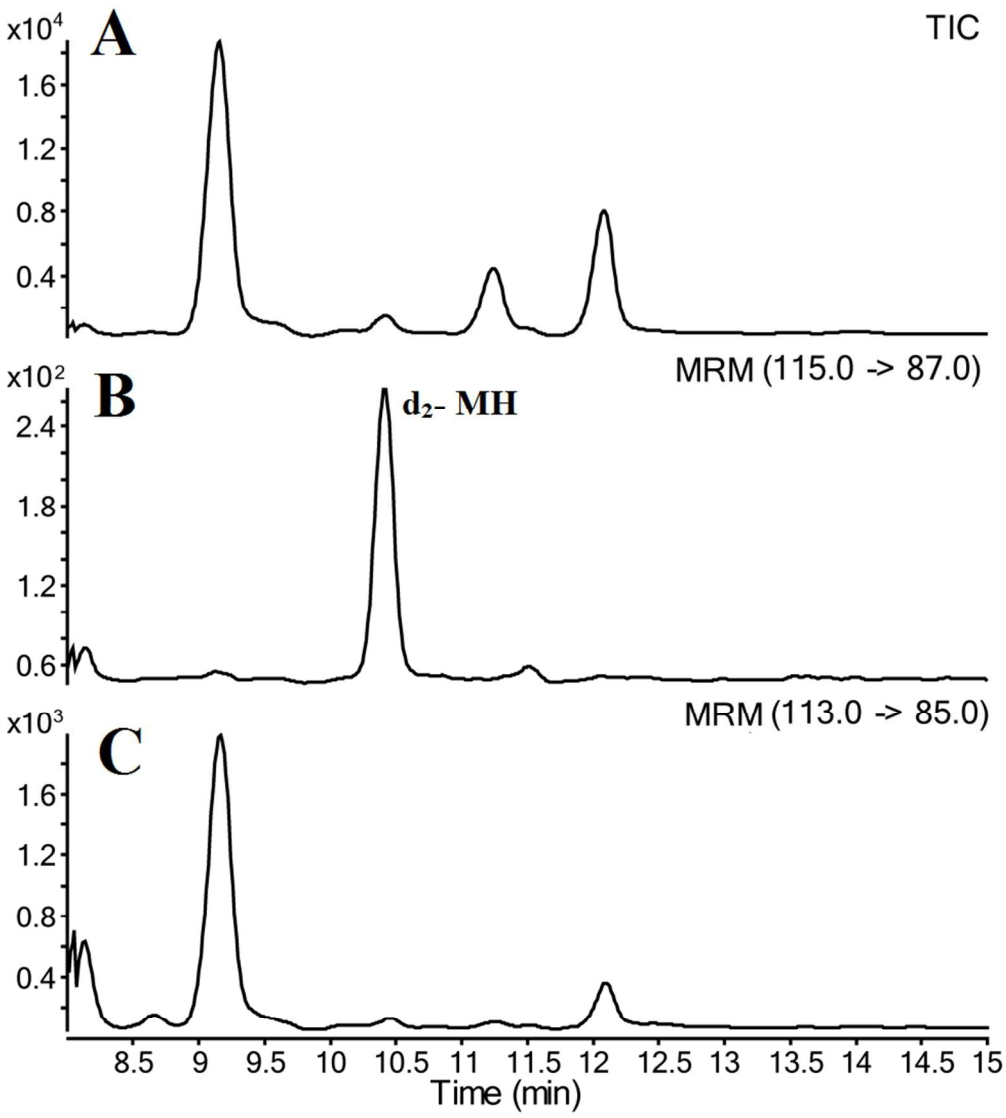

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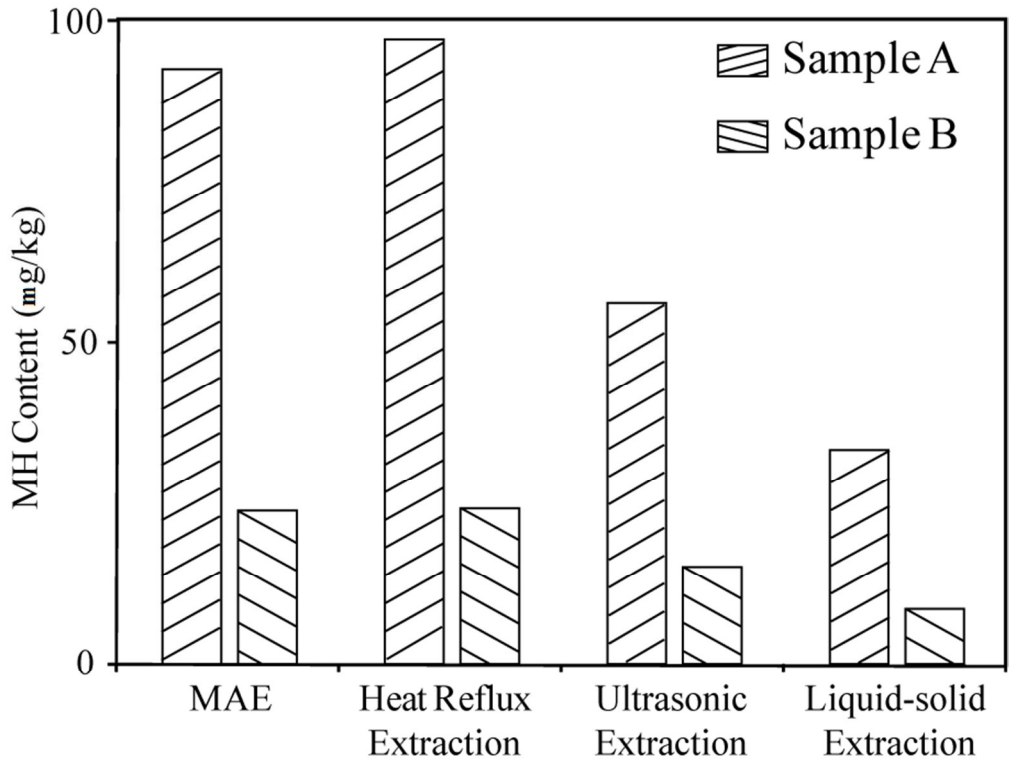

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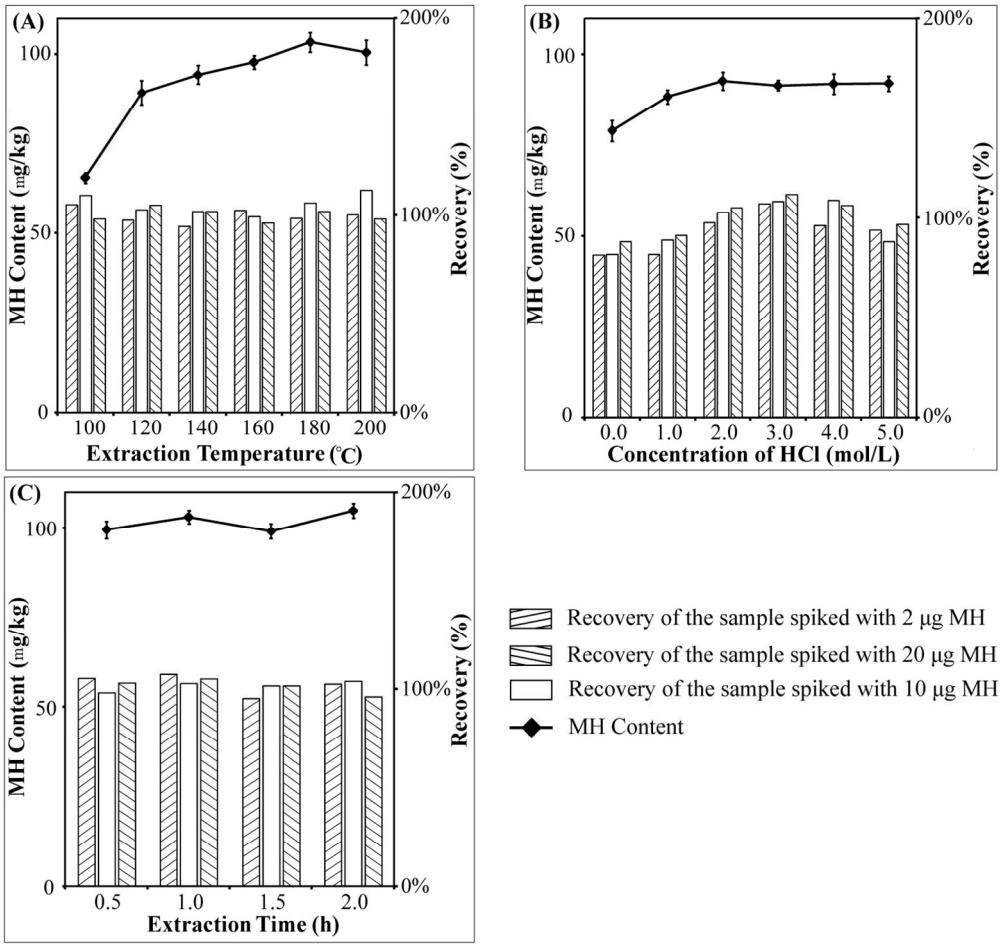

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