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Preconcentration of Heterocyclic Aromatic Amines in Edible Fried Insects Using Surfactant-Assisted Hydrophobic Deep Eutectic Solvent for Homogeneous Liquid−Liquid Microextraction prior to HPLC

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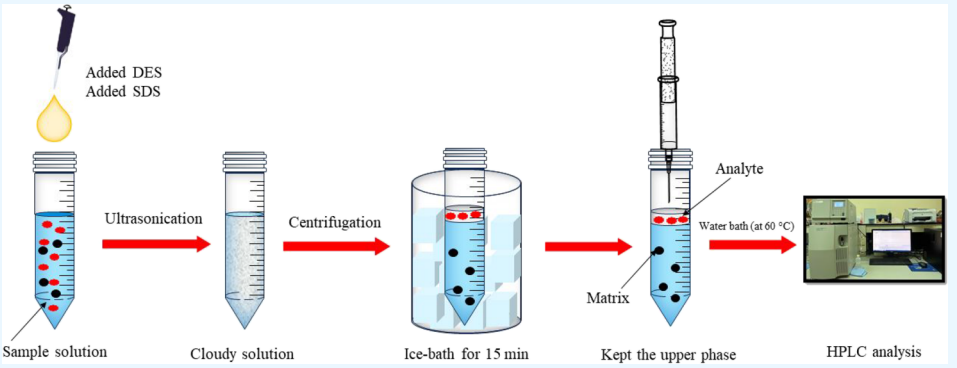

ABSTRACT: Thermal processing techniques are often accompanied by the production of many harmful compounds such as heterocyclic aromatic amines (HAAs). To protect human health, an efficient and environmentally friendly method, namely, homogeneous liquid−liquid microextraction (HLLME), was investigated. This method is based on a surfactant-assisted hydrophobic deep eutectic solvent for the determination of HAAs in edible fried insect samples prior to their analysis by high-performance liquid chromatography coupled with UV detection. A hydrophobic deep eutectic solvent (as extraction solvent) was synthesized using decanoic acid as a hydrogen bond donor and tetrabutylammonium bromide (TBABr) as a hydrogen bond acceptor and then characterized by Fourier transform infrared (FTIR) spectroscopy. The surfactant was used as the emulsifier and induces mass transfer, resulting in an increasing extraction efficiency of the proposed method. Various factors affecting the extraction performance were investigated and optimized. A matrix-match calibration method was used to analyze HAAs in high heat-treated edible fried insect samples. Under optimized conditions, the proposed method showed good linearity ($R^2 \geq 0.99$) with satisfactory limits of detection and satisfactory reproducibility with relative standard deviation of less than 10.0%. Furthermore, the procedure greenness was assessed using the Analytical Eco-Scale. This paper represents the first application of HLLME based on a surfactant-assisted hydrophobic deep eutectic solvent to analyze HAAs in edible fried insect samples.

1. INTRODUCTION

Recently, edible insects are gaining increasing attention as trendy foods and are *inter alia* discussed as novel alternative protein sources for human food and animal feed.[1] As most existing literature indicates, edible insects are considered a sustainable and economical food source.[2] Currently, insect-based foods are customized for western palates by developing new food products via traditional (e.g., roasting and defatting) insect flours and novel (e.g., enzymatic proteolysis and sonication) processing techniques that allow for insects to be used as protein-rich ingredients in food formulation.[3−5] Most edible insects are either fried, barbecued, dried and ground, or steamed in banana leaves and curried.[6] Thermal processing techniques (including grilling, frying, smoking, and boiling) not only can destroy pathogenic microorganisms but also endow foods with attractive colors, fragrances, and flavors. Deep frying is among the most common thermal processing

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methods for edible insect cooking. Eating edible fried insects is popular in the northern and northeastern parts of Thailand. However, this process can also be accompanied by the production of many harmful compounds, such as acrylamide (AA), heterocyclic aromatic amines (HAAs), 5-hydroxymethylfurfural (5-HMF), and advanced glycation end products.[7] HAAs are compounds produced via the pyrolysis of proteins and amino acids.[8] 2-Amino-3-methyl-3*H*-imidazo[4,5-*f*]-quinoline (IQ) was grouped as a class 2A compound (probable carcinogen for humans), while other types, including 2-amino-3,4-dimethyl-imidazo[4,5-*f*]-quinoline (MeIQ), amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-9*H*-pyrido[3,4-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indol (MeAαC), and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]-indole (Trp-P-2) were classified as class 2B compounds (possible human carcinogens) by the International Agency for Research on Cancer (IARC).[9] Therefore, determination and monitoring of HAAs in heat processing are of great significance for food safety and human health.

Owing to trace levels of HAAs and the interferences of complex matrices, sample preparations are essential before instrumental analysis.[10] Various sample preparation methods were used in order to enrich trace amounts of aromatic amines, e.g., liquid−liquid extraction,[11] solid-phase extraction (SPE),[12,13] solid-phase microextraction (SPME),[14] dispersive liquid−liquid microextraction (DLLME),[15] magnetic solid-phase extraction,[9] ion-pair-based surfactant-assisted dispersive liquid−liquid microextraction,[16] and air-agitated cloud-point extraction.[17]

Homogeneous liquid−liquid microextraction (HLLME), as a kind of novel and powerful pretreatment technique, has attracted wide attention because of rapid extraction, simple operation, and desirable enrichment factors.[18] The phase separation of a homogeneous solution and the mass transfer of organic compounds into an extractant phase occur concurrently in HLLME.[19] In addition, a rapid extraction equilibrium is achieved due to the extremely large contact surface between the sample and extractant phases.[20] HLLME provides several merits, including ease of operation, rapidity, and less auxiliary equipment,[21] but it still has disadvantages involving the use of toxic extraction solvents and inconvenient collection of the extractant phase.[22] Therefore, developing an environmentally friendly and cost-effective solvent is of utmost importance in chemical manufacturing.[23] Nowadays, alternative solvent is used, namely, deep eutectic solvents (DESs). They are known as green solvents which could be used as extraction solvents instead of the common toxic organic solvents[24] such as carbon tetrachloride and dichloromethane. DESs can be easily synthesized by mixing a hydrogen bond acceptor (HBA) and one or two hydrogen bond donor (HBD).[25−30] A wide range of HBDs and HBAs have been used to make DESs. These are composed of a mixture of safe, cheap, renewable, and biodegradable organic compounds that are capable of associating with each other through hydrogen bonding and forming a compound that has a melting point far below that of either component.[31] Therefore, DESs have properties such as ease of synthesis, low vapor pressure, low toxicity, high thermal stability, inexpensiveness, biocompatibility, and renewability.[32] DES as an extraction solvent must have some characteristics such as high extraction affinity to the analytes, low solubility in aqueous solution, and easy dispersion into water.[31] Moreover, they have unique properties such as high purity and environmental friendliness.[33] DES was applied for the extraction of neonicotinoid insecticides,[34] triazole fungicides,[23] curcumin,[35] caffeine,[36] and antibiotics.[22]

Therefore, the main objective of this study was to investigate an effective analytical method for the preconcentration of HAAs in edible fried insects using surfactant-assisted hydrophobic DES for HLLME prior to HPLC. Various extraction parameters affecting the extraction efficiency were investigated, and the proposed method was applied to analyze complex sample matrices. The proposed method was compared with other extraction methods previously reported. Moreover, to our knowledge, this is the first time that a surfactant-assisted hydrophobic DES is used for HLLME for the extraction of HAAs in edible fried insects.

2. RESULTS AND DISCUSSION

2.1. Characterization of Hydrophobic DESs. To characterize the formation of the hydrophobic DES and its important functional groups, attenuated total resistance-Fourier transform infrared (ATR-FTIR) spectrometry was utilized, as shown in Figure 1. The formation of hydrogen

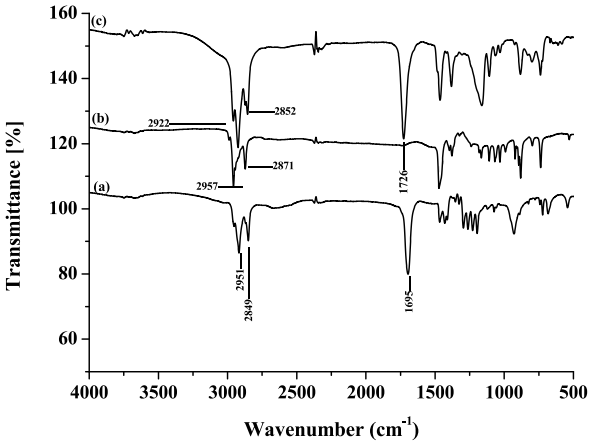

Figure 1. FTIR spectra of (a) decanoic acid, (b) TBABr, and (c) hydrophobic DESs.

bonds between decanoic acid as a HBD and tetrabutylammonium bromide (TBABr) as a HBA is accomplished. Decanoic acid [as shown in Figure 1a] shows characteristic bands at 2951 cm^{-1} (O−H stretching), 1695 cm^{-1} (C═O stretching), and 2849 cm^{-1} (−CH_3 stretching). FTIR spectra of TBABr [as shown in Figure 1b] reveal methylene group (−CH_2) and methyl group (−CH_3) bands at wavenumbers 2871 and 2957 cm^{-1}, respectively. Moreover, the spectra of DESs [as shown in Figure 1c] shift to 1726 cm^{-1}, which is characteristic of the stretching vibrations of the carbonyl group and indicates the formation of new hydrogen bonds in the vicinity of the COOH group. Thus, the shift of the O−H vibrations proves the formation of a hydrogen bond between components and, as a result, demonstrates the DES formation.

2.2. Optimization of HLLME Using Surfactant-Assisted Hydrophobic DES Conditions. In order to obtain high and stable extraction efficiencies, the main factors affecting extraction were investigated and optimized. These include the composition of the DES solvent, the volume of hydrophobic DES, the kind and concentration of the surfactant, the sample volume, the ultrasonication extraction temperature and time, and the salt addition. The study was performed using an aqueous standard containing the analytes

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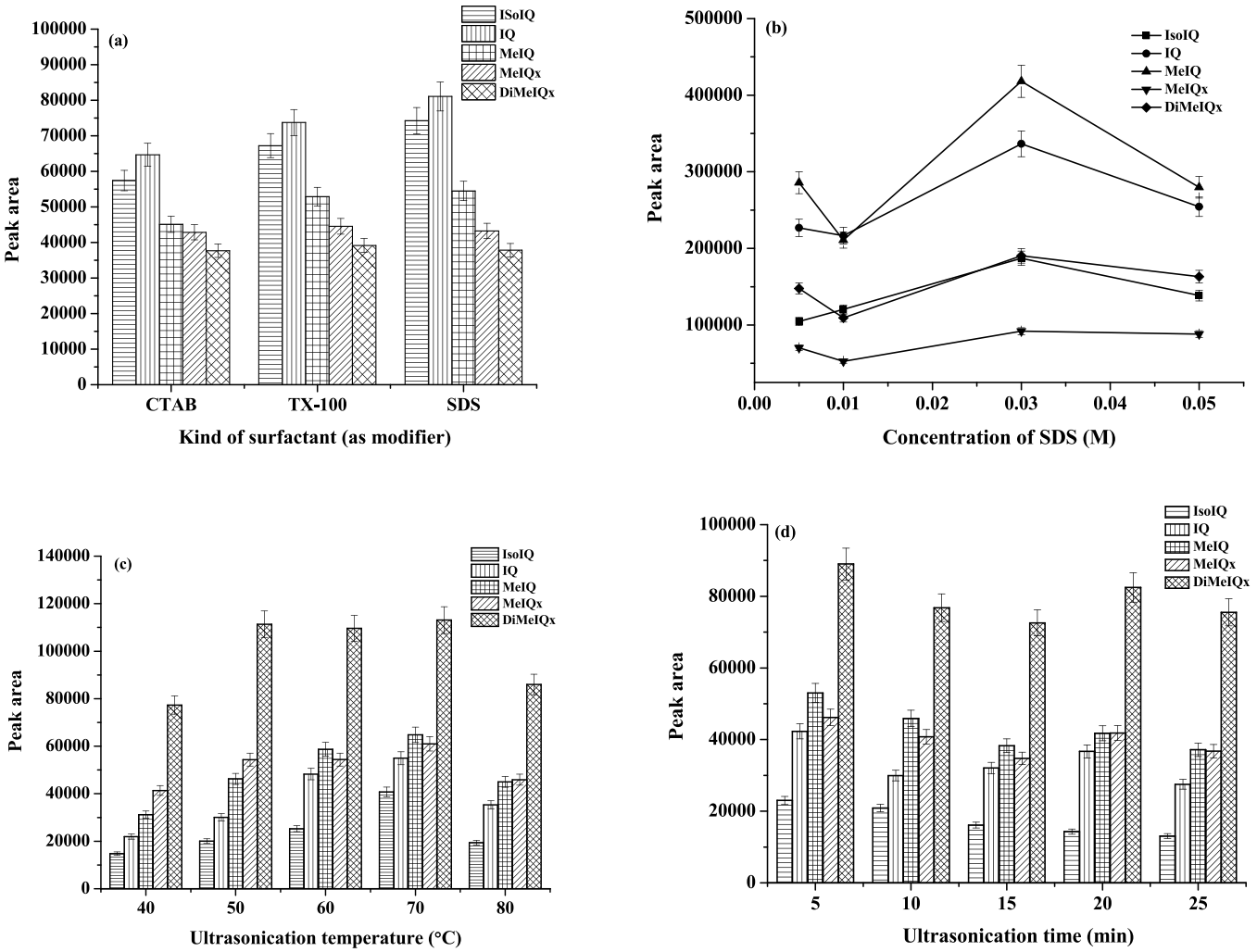

Figure 2. Effect of the (a) kind of surfactant (as a modifier), (b) concentration of SDS (M), (c) ultrasonication temperature, and (d) ultrasonication time on the extraction efficiency.

at 100 μg L^{-1}. Analyses were carried out in triplicate in all cases.

The composition of DES solvents is important for electrostatic interaction, selectivity, and efficient separation with the analyte.[35] In this present work, decanoic acid with TBABr were used for preparation of hydrophobic DES. Four different mole ratios of hydrophobic DES were studied, including 1:1, 2:1, 3:1, and 4:1 (data not shown). It was found that DES at the mole ratio of 1:1 provided higher extraction efficiency in terms of peak area because of low viscosity. The low viscosity of DES leads to high diffusivity,[37] which can improve the extraction efficiency of the target analytes. At the other mole ratios (2:1, 3:1, and 4:1), the separation phase was sticky and viscous, which could not be injected into the HPLC system. Consequently, hydrophobic DES at a mole ratio of 1:1 was selected.

The volume of hydrophobic DES has a large influence on the extraction efficiency. The effect of different volumes of hydrophobic DES (20, 50, 100, 150, and 200 μL) on the peak area of analytes extracted was investigated to obtain the best extraction conditions (data not shown). Larger volumes of hydrophobic DES (>100 μL) did not increase the extraction efficiencies of HAAs because of the dilution effect. Lower volumes of hydrophobic DES (less than 100 μL) did not result in phase separation. After careful consideration, a volume of hydrophobic DES (100 μL) was chosen for HAAs.

The properties of surfactants suggest that they may improve the extraction efficiency because the surfactant structure influences its physical and chemical properties.[38] Therefore, the addition of the surfactant also considerably influenced the proposed microextraction procedure. Various kinds of surfactants including cationic surfactant [cetyltrimethylammonium bromide (CTAB)], nonionic surfactant [Triton X-100 (TX-100)], and anionic surfactant [sodium dodecyl sulfate (SDS)] at 0.03 M for all surfactants were studied as a disperser solvent. The highest extraction efficiency in terms of peak was obtained when SDS was added [as shown in Figure 2a] because a surfactant aggregate orients its hydrocarbon tails toward the center to create a nonpolar core. When the hydrophobic substances were separated, it is favorably partitioned in the hydrophobic core of micelles.[39] Thus, SDS was selected in this study. The effect of concentration of SDS was also studied in the range of 0.005−0.05 M [as shown in Figure 3b]. The results show that the peak areas of all analytes increased by increasing the SDS concentration up to 0.03 M. Therefore, the concentration of SDS 0f 0.03 M was used for further studies.

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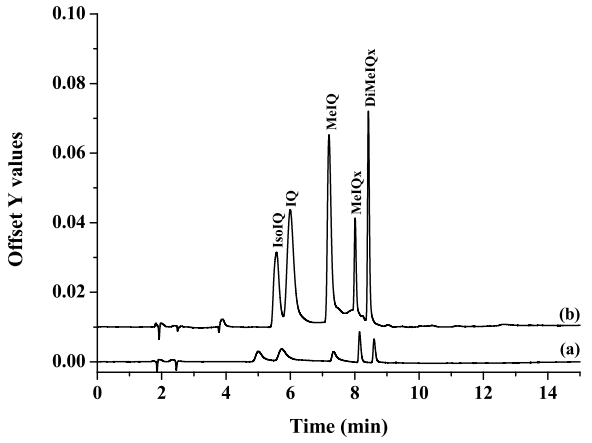

Figure 3. Chromatograms of standard HAAs obtained (a) without preconcentration and (b) with preconcentration using the proposed HLLME using the surfactant-assisted hydrophobic DES procedure.

Sample volume is one of the most important factors to be investigated because it determines the enrichment factor of the method. The effect of sample volume was examined in the range of 5.00−20.00 mL. The peak areas of the analytes increased with the increase in the sample volume from 5.00 to 10.00 mL and then slightly reduced with the further increase in the sample volume to 20.00 mL. Therefore, the sample volume of 10.00 mL was selected for further studies.

Addition of salt to an aqueous sample enhanced the availability of analyte for extraction while the volume of extractant obtained is increased, resulting in a decrease in both the target analyte concentration and the enrichment factor.[40] It was evaluated considering various concentrations (expressed as percentage) of NaCl in the range of 0 to 5.0% (data not shown). It was found that the addition of various salts could not maintain phase separation. Therefore, this study was performed without salt addition.

To decrease the viscosity of the extract before injecting it into the HPLC-UV system, an ultrasound agitator was used without the use of the dissolving solvent. Various ultrasonication temperatures were studied including 40, 50, 60, 70, and 80 °C [as shown in Figure 2c]. The results show that the peak areas of all analytes increased by increasing the ultrasonication temperature up to 70 °C and then decreased due to the decomposition of molecules at higher temperature. Therefore, an ultrasonication temperature up to 70 °C was adopted for the HPLC-UV system without the use of the dissolving solvent.

The ultrasonication extraction time was evaluated from 1 to 25 min. For the ultrasonication extraction time of 1 min, no phase separation occurred. The results, which are shown in Figure 2d, indicate that the signal decreased with an increase in the extraction time. The ultrasonication extraction time of 5 min was selected as the optimum value since it provides a good sensitivity level and a good sample throughput and several samples can be simultaneously extracted in the orbital shaker.

2.3. Analytical Performance and Method Validation. The analytical performance of the method was determined under optimized conditions to evaluate the performance of HLLME. The results, such as calibration curve, determination coefficients (R^2), enrichment factors (EFs), limit of detection (LOD), limit of quantification (LOQ), and relative standard deviations (RSDs), are presented in Table 1.

The calibration standards were analyzed at seven different concentrations and were achieved over the concentration range of 60−1000 μg L^{-1} for IsoIQ, IQ, and MeIQ and 0.9−30 μg L^{-1} for MeIQx and DiMeIQx. The sensitivity was evaluated in terms of LOD as the concentration giving the signal-to-noise ratio of 3 (S/N = 3) and ranged between 0.3 and 20 μg L^{-1}, while LOQ (S/N = 10) was from 0.9 to 60 μg L^{-1}. The precision of the proposed method was invented by testing interday and intraday variations. The interday was evaluated by five replicates at two concentrations (50 and 100 μg L^{-1}) in the same day, and the obtained RSD values were less than 9.55%. The intraday precision was measured on three ensuring days with RSD values less than 9.94%. Good precision with RSDs was less than 10.0%. The EF was determined by comparison of the concentration ratio of the analytes in the settled phase (C_{set}); later, the sedimented phase is melted in an ultrasonic bath (70 °C) and in the aqueous sample (C_o) with values between 2.28 and 26.3 folds depending on the compounds. Chromatograms of the studied HAAs obtained from direct HPLC and preconcentrated by the proposed microextraction method are shown in Figure 3. After the microextraction method, the chromatographic signals were improved.

2.4. Application to Real Samples. In order to investigate its potential practical application, the optimized HLLME using a surfactant-assisted hydrophobic DES was used for extraction of the HAAs from edible fried insect samples. The matrix-match calibration was used for quantitation of the target analytes in the edible fried insect samples. Matrix-match calibration data obtained in edible fried insect samples are listed in Table 2. The working linear range was 60−1000 μg kg^{-1} for IsoIQ, IQ, and MeIQ and 0.9−30 μg kg^{-1} for MeIQx and DiMeIQx with the coefficient for determination greater

Table 1. Analytical Features of the Proposed Method for the Determination of HAAs[a]

						intraday precision (n = 5), RSD (%)		interday precision (n = 5), RSD (%)		
analyte	linear range (μg L^{-1})	linear equation	R^2	LOD (μg L^{-1})	LOQ (μg L^{-1})	t_R	peak area	t_R	peak area	EF (C_{ex}/C_o)[b]
IsoIQ	60−1000	y = 182,964x + 1514.5	0.9971	20	60	0.46	9.55	2.75	9.94	2.28
IQ	60−1000	y = 387,277x + 2001.5	0.9947	20	60	0.57	5.54	3.63	5.96	3.66
MeIQ	60−1000	y = 411,531x + 14733	0.9969	20	60	0.95	3.53	1.88	5.75	6.51
MeIQx	0.9−30	y = (2 × 10^6)x − 1632.7	0.9913	0.3	0.9	0.44	5.19	1.04	3.23	9.49
DiMeIQx	0.9−30	y = (4 × 10^6)x − 5632.9	0.9956	0.3	0.9	0.56	1.13	0.27	4.20	26.31

[a]Precision was calculated at the concentration of 50 and 100 μg L^{-1} for each triazole. [b]Ratio of the concentration of the target analytes in the extraction phase to the initial concentration in the aqueous sample.

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Table 2. Matrix-Match Calibrations of Heterocyclic Amines in Edible Fried Insect Samples (n = 3)

analyte	IsoIQ linear equation (R^2)	IQ linear equation (R^2)	MeIQ linear equation (R^2)	MeIQx linear equation (R^2)	DiMeIQx linear equation (R^2)
grasshoppers	y = 17,1927x + 1617.2 (0.9951)	y = 407,277x + 1701.5 (0.9978)	y = 421,637x + 22,415 (0.9980)	$y = (2 \times 10^6)x$ − 2232 (0.9940)	$y = (4 \times 10^6)x$ − 5647 (0.9955)
crickets	y = 175,931x + 1510 (0.9945)	y = 391,545x + 1211 (0.9940)	y = 388,411x + 24,110 (0.9978)	$y = (2 \times 10^6)x$ − 4232 (0.9955)	$y = (4 \times 10^6)x$ − 5674 (0.9977)
chrysalis	y = 162,634x + 1812 (0.9950)	y = 394,461x + 1114 (0.9978)	y = 414,632x + 24,110 (0.9955)	$y = (2 \times 10^6)x$ − 4132 (0.9955)	$y = (4 \times 10^6)x$ − 6457 (0.9955)
bamboo caterpillar	y = 168,218x + 1916 (0.9950)	y = 398,124x + 1310(0.9955)	y = 413,611x + 14231 (0.9920)	$y = (2 \times 10^6)x$ − 2846 (0.9960)	$y = (4 \times 10^6)x$ − 5432 (0.9952)
Mole cricket	y = 172,218x + 1916 (0.9940)	y = 388,124x + 1210 (0.9945)	y = 403,611x + 15,231 (0.9927)	$y = (2 \times 10^6)x$ − 3032 (0.9956)	$y = (4 \times 10^6)x$ − 6232 (0.9940)

Table 3. Comparison with Other Reported Methods for the Determination of HAAs[a]

method	analytes	sample	analytical technique	LOD	recovery (%)	ref.
microwave-assisted extraction-DLLME	IQ, MeIQ, MeIQx	hamburger	HPLC/PDA	0.06−0.21 μg kg^{-1}	90−105	41
hollow fiber membrane liquid-phase microextraction	MeIQx, PhIP, 4,8-DiMeIQx, 7,8-DiMeIQx	blood	LC-MS/MS	2−5 pg mL^{-1}	92.0−99.4	42
air-agitated cloud-point extraction	MeIQ, 4,8-DiMeIQx, PhIP, harmane	smoked sausage samples	HPLC/PDA	0.001−0.003 mg kg^{-1}	89.7−103.8	17
SPME (ionic liquid as desorption solvent)	Six HAAs	meat samples	HPLC/FL	0.30−75 ng L^{-1}	68.5−118	43
ion-pair-based surfactant-assisted DLLME	MeIQ, 4,8-DiMeIQx, PhIP, Harmane	grilled pork	HPLC/PDA	0.01 μg kg^{-1} for all compounds	90−106	16
HLLME using surfactant-assisted hydrophobic DES	IQ, IsoIQ, MeIQ, MeIQx, DiMeIQx	edible fried insects	HPLC/PDA	0.3−20 μg kg^{-1}	90−106	this study

[a]NR: not reported.

than 0.99. The obtained LODs were in the range of 0.3 to 20 μg kg^{-1}. For the edible fried insect samples studied, two HAAs (MeIQx and DiMeIQx) were detected in the range of 1.0−3.0 μg kg^{-1} (data not shown).

To demonstrate the capability of the developed method, the edible fried insect samples were spiked with standard HAAs at three concentration levels of 60, 100, and 150 μg kg^{-1} for IsoIQ, IQ, and MeIQ and 0.9, 20, and 50 μg kg^{-1} for MeIQx and DiMeIQx before analysis. All experiments were performed in triplicate. It was found that, the recoveries of the studied HAAs were between 90 and 106% (data not shown) with RSD less than 7.6% of all samples, respectively. The results demonstrated that the proposed method based on HLLME using surfactant-assisted hydrophobic DES coupled with HPLC is accurate and dependable for the microextraction method and for the determination of HAAs in edible fried insect samples.

2.5. Comparison of the Proposed Method to Other Related Methods. To assess the analytical performance of the proposed method, a comprehensive comparison of the developed method in this study with other reported methods[16,17,41−43] for the analysis of HAAs was conducted (as shown in Table 3). As can be seen, the proposed method showed a relatively higher extraction recovery. It can be clearly observed that the extraction time of this method is much shorter than those for other methods. In addition, assistant means such as ultrasonication or centrifugation were both needed in this method to attain phase separation and extraction. All of these results indicate that HLLME using surfactant-assisted hydrophobic DES is a reproducible, rapid, simple, and low-cost technique, which can be used for preconcentration of HAAs in edible fried insect samples.

2.6. Greenness Evaluation. Greenness of the method was investigated using Analytical Eco-Scale (AES) factors. Based on AES parameters, the method of greenness was considered by calculating penalty points (PPs) according to the following formula: AEC = 100 − total PPs.[44] Many parameters were considered for the calculation of AEC score in this methodology, including the amount of chemicals used, energy consumption, potential hazards, and waste production. The scores >75 represented excellent green analysis, while the scores between 50 and 75 are acceptable green analysis, and the scores <50 are inadequate green analysis.[45] The total PPs consist of amount PP x hHazard PP called subtotal PP. These values were calculated in accordance with The Globally Harmonized System of Classification and Labeling of Chemicals (GHS) criteria that evaluated physical, environmental and health hazards. The PPs in the GHS system were easily introduced by the standard hazard pictograms and signal word as danger PPs = 2 and warning PPs = 1. The PPs of energy for laboratory practices and instrumental analyses assigned based on energy consumption of ≤0.1, ≤1.5, and >1.5 kWh were 0, 1, and 2 PPs, respectively.[44] As shown in Table 4, the final AEC score for this proposed method is 80, which indicates that the developed method can be considered as excellent greenness analysis.

3. CONCLUSIONS

A simple and effective method, namely HLLME using surfactant-assisted hydrophobic DES has been investigated and applied to the determination of HAAs in edible fried insects prior to HPLC. The proposed approach has shown favorable linearity, enrichment factors, LODs and LOQs, extraction recoveries, and RSDs. Considering the complexity of the matrix samples, the extraction methodology is reproducible

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Table 4. PPs for Hydrophobic DES-Based HLLME Procedure

parameter	penalty points
amount of sample (10 mL)	2
hydrophobic DES synthesis	
-decanoic acid	1
-TBABr	5
-heating <1 h	1
HLLME procedure	
-hydrophobic DES (0.1 mL)	0
-SDS (0.03M, 0.1 mL)	0
ultrasonic dispersion	2
hazard (physical, environment, health)	1
energy (≤1.5 kWh per sample)	1
occupational hazard	0
waste (1−10 mL)	3
no treatment	3
FTIR (≤0.1 kWh per sample)	0
UV−vis spectrometry (≤0.1 kWh per sample)	0
LC (≤1.5 kWh per sample)	1
PPs total:	20
eco-scale	80

and fast. The proposed method has the potential to be used as an alternative extraction method for the determination of HAAs in edible fried insect samples. Moreover, in its present form, the method reaches the legal limits imposed by the US Environmental Protection Agency. The assessment of greenness of the method by the AES indicated a score of 80 out of 100 and was evaluated as an acceptable green procedure.

4. EXPERIMENTAL SECTION

4.1. Chemicals and Reagents. Methanol and acetonitrile of HPLC grade were supplied by Merck (Darmstadt, Germany). Acetic acid was purchased from Fluka, Germany. Triton X-100 and SDS were purchased from Merck, Germany. CTAB was obtained from Calbiochem, Germany. Decanoic acid was obtained from Sigma-Aldrich, Germany. TBABr was purchased from ACROS Organics, USA. Five HAA standards, including IQ, 2-amino-1-methylimidazo[4,5-*f*]quinoline (IsoIQ), 2-amino-3,4-dimethyl-3*H*-imidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), and 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]-quinoxaline (DiMeIQx), with a purity of ≥99% were obtained from Santa Cruz Biotechnology, Canada. Each compound's standard stock solutions (1000 μg L^{-1}) was prepared by dissolution of proper amounts of HAA in methanol and stored at 4 °C. The working standard solutions were prepared by diluting the stock solution with water.

Deionized water (Millipore Waters, USA) with a resistivity of 18.2 MΩ cm was used throughout the experiments.

4.2. Instruments and Chromatographic Separation. Separation of HAAs was performed using a Waters liquid chromatograph (Waters, USA) equipped with a 1525 Binary HPLC pump, a Rheodyne injector with a sample loop of 20 μL, and a diode array detector. Empower 3 software (Waters, Milford, USA) was used for operation and data acquisition. A Purosphere STAR RP-18 end-capped column (150 × 4.6 mm, 5.0 μm) was used as an analytical column operating at room temperature. Gradient elution of acetonitrile (solvent A) and 2% (v/v) acetic acid (solvent B) was carried out at a flow rate of 1.0 mL min^{-1}. The gradient program was as follows: 0−7 min, 93% B; 6−8 min, 80% B and increased linearly to 85% B in 2 min. Then, it increased linearly to 93% B, and it was held for 3 min. The detection wavelength for HAAs was set at 260 nm.

Extention process of the functional group after surface modification was performed with a Bruker INVENIO-S FTIR spectrometer (Bruker Corp., Massachusetts, USA) at 4000−400 cm^{-1} using diamond lens ATR. Phase separation was completed by a centrifuge agitator (Centurion, England). A vortex mixer (Fisher Scientific, USA) was also used.

4.3. Synthesis of the Hydrophobic DES. Hydrophobic DESs were synthesized by using decanoic acid mixed with TBABr at different molar ratios (1:1, 2:1, 3:1, and 4:1). The synthesized samples were heated at 80 °C in water bath for an hour until transparent clear liquids were formed. The prepared DESs and the DESs collected after the phase transition were characterized by FTIR spectrometry using diamond lens ATR to examine their formation and chemical structure. The newly formed DES remains liquid at room temperature until used.

4.4. HLLME Using Surfactant-Assisted Hydrophobic DES. Figure 4 presents a schematic illustration of the HLLME procedure. A 10.00 mL sample was added to a centrifuge tube. In the first HLLME, 100 μL of DES and 0.003 M (100 μL) SDS were added successively. The mixture was subjected to ultrasound at 70 °C for 5 min and centrifugation at 3500 rpm

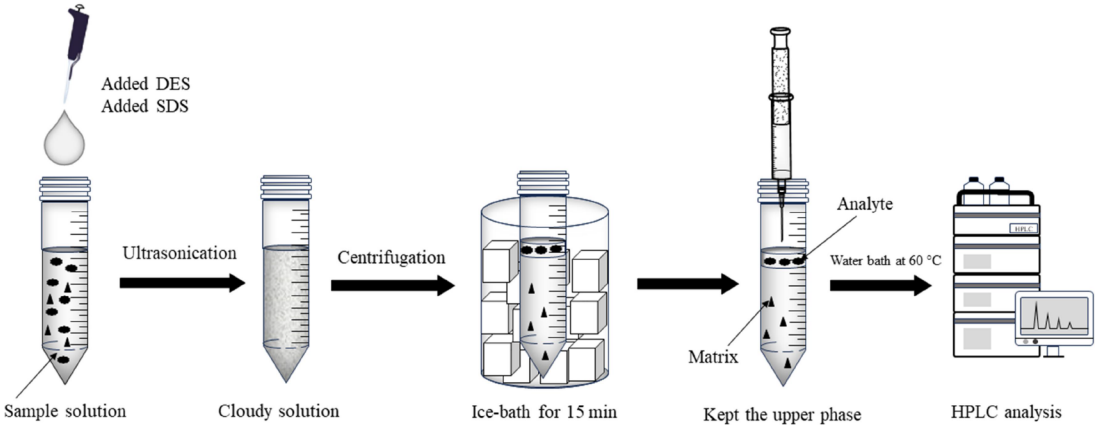

Figure 4. Schematic illustration of HLLME using a surfactant-assisted hydrophobic DES procedure (photograph courtesy of "Wannipha Khiaophong". Copyright 2023).

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for 5 min. Then, the mixture was left in an ice bath for 5 min to solidify the extraction solvents in the upper part of the tube. The collected extractant phase was transferred and melted in an ultrasonic bath (70 °C) before subsequent HPLC analysis.

4.5. Sample Preparation. Five edible fried insects, namely, the grasshoppers, crickets (orthoptera), silk worm pupa (*Bombyx mori*), bamboo caterpillar (*Omphisa* sp.), and mole cricket (*Gryllotalpa* sp.), were collected from the local market in Kantharawichai District, Maha Sarakham province, Northeast, Thailand. To prepare the edible fried insect samples, 1.0 g of spiked and nonspiked edible fried insect samples were sonicated for 5 min at 45 °C with 3 mL of extractant phase (2% acetic acid in acetonitrile) in an ultrasonic bath with a fixed power. Then, the supernatant was separated by centrifugation (20 min, 4000 rpm). The solid residue was subjected to a second extraction with 3 mL of the extractant phase. Once all volumes of extractant phase were gathered, the funnel was placed in the freezer for 1 h at −18 °C to separate the fat of the sample. Approximately 5 mL were decanted and followed by centrifugation. An aliquot was used for HLLME under the described conditions and analyzed by an HPLC-UV system. For the accuracy of evaluation, the studied edible fried insect samples were spiked with HAAs at three different concentration levels prior to the extraction and preconcentration steps.

4.6. Evaluation of EF and Relative Recovery. In order to study the effect of experimental extraction conditions on the extraction efficiency, EF was evaluated between the analyte concentration in the final phase (C_{sed}) and the initial concentration in the analyte in aqueous sample solution (C_0) according to eq 1:

$$EF = C_{sed}/C_0 \quad (1)$$

The percentage relative recovery (RR, %) was calculated as the % amount of analyte recovered from the matrix with reference to the extracted standard (standard spiked into the same matrix) according to eq 2:

$$RR(\%) = \frac{C_{found} - C_{real}}{C_{added}} \times 100 \quad (2)$$

where C_{found} is the concentration of the analyte after adding a known amount of working standard to the real sample, C_{real} is the analyte concentration in the real sample, and C_{added} represents the concentration of a known amount of working standard that was spiked into the real samples.

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The authors declare no competing financial interest.

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