# DETERMINATION OF NICOTINE AND ITS METABOLITES ACCUMULATED IN FISH TISSUE USING QUECHERS AND HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

by

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Abstract

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The quantitative determination of nicotine and its major metabolites (cotinine and anabasine) in fish tissue was performed using hydrophilic interaction liquid chromatography (HILIC) coupled with tandem mass spectrometry. Marine and freshwater fish were purchased from local grocery stores and were prepared based on the well-known QuEChERS (quick, easy, cheap, effective, rugged, safe) sample preparation protocol. There were significant suppressions on measured nicotine signals (10%) due to the matrix effects from marine fish, but no obvious effects on freshwater fish signals. Method validation was incorporated with internal standards and carried out with matrix-matched calibration. The detection limits for nicotine, cotinine, and anabasine were 9.4, 3.0, and 1.5 ng/g in fish, respectively. Precision was acceptable and less than 9% RSD at low, mid, and high concentrations. Acceptable and reproducible extraction recoveries (70 – 120%) of all three compounds were achieved, except for anabasine at low concentration (61%). This developed method offers a fast, easy, and sensitive way to evaluate nicotine and its metabolite in fish tissues.

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# Chapter 1

# Introduction and Background

## 1.1 Nicotine in the environment

Nicotine has been widely used as a botanical insecticide in the US, Canada, and other parts of the world for centuries. As with many pesticides, pollution of the environment is a concern. In January 1, 2014, the Environmental Protection Agency (EPA) outlawed the use of nicotine insecticide products in the US [1]. The plant alkaloid nicotine is a basic compound composed of pyridine and pyrrolidine rings (heterocyclic amines). It is a poisonous and pharmacologically active substance found in tobacco [2-4]. Metabolism of nicotine in human beings yields cotinine as the major product. Anabasine can also be found in tobacco products, which has been used along with cotinine as a marker of smokers in nicotine replacement therapies [5].

Nicotine accumulation and its toxicity in aquatic organisms is a major concern in the environment [6]. Konar [7] evaluated the toxicity of nicotine to aquatic life by exposing several aquatic organisms to different levels of nicotine concentrations. The results showed that median lethal concentrations ($LC_{50}$) for most fish species were about 2.5 ppm for a 168-hour nicotine exposure. Combining the usage of nicotine activators (sodium carbonate and lime) and nicotine exposure (concentrations ranging from 0.5 to 5 ppm), all fish species died within 13 hours. Slaughter et al. [8] evaluated the other possible contamination sources of nicotine, cigarette butts, using EPA standard acute fish bioassays. They found that cigarette butts were acutely toxic to marine and freshwater fish, especially for the smoked cigarette butts accompanied with tobacco. $LC_{50}$ for both were within 1.1 cigarette butts/L at a 96-hour exposure.

## 1.2 Hydrophilic interaction liquid chromatography

Hydrophilic interaction liquid chromatography (HILIC) proposed by Alpert [9] in 1990 has been applied for analysis of high polarity and hydrophilic compounds. The continuing improvements in HILIC mode separations have led to many new applications in metabolomics [10], drug monitoring [11], proteomics [12], environmental analysis [13], and other fields. HILIC has the advantage of enhanced detection sensitivity when coupled with mass spectrometry due to its high organic content eluting solvent, which also provides low column backpressure because of low viscosity mobile phase. It enhances the electrospray ionization efficiency and desolvation process so as to facilitate the compatibility with mass spectrometry. HILIC features the use of polar stationary phase, such as diol, silica, cyano, and amino phases, among others. In spite of reversed-phase liquid chromatography (RPLC) being the most widely applied separation technique, HILIC can be a complementary alternative to separate highly polar compounds that are not retained or elute too early in RPLC. Mobile phases in HILIC are very similar to RPLC. They incorporate similar types of buffers and concentrations, pH values, and organic modifiers [14]. In general, analytes are eluted in order of increasing hydrophilicity, opposite to RPLC analyte retention; in HILIC, acetonitrile is the weak solvent and water (aqueous) is the strong solvent in the mobile phase.

There are two general accepted ways to describe the mixed mode mechanism of HILIC retention. These include adsorption of analytes directly on the stationary phase and/or partitioning of analytes between a water-enriched layer on the polar surface of stationary phase and a predominantly organic mobile phase [15]. HILIC separation often occurs through mixed modes of interactions, including hydrogen bonding, electrostatic, dipole-dipole, hydrophilic, and even hydrophobic interactions. Comparing all the interactions above, hydrogen bonding interaction appears to play the most important role

in the adsorption retention mechanism [16]. Commercial HILIC columns can be classified into several types: bare silica, neutral phases, charged phases, and zwitterionic phases. They differ significantly in their retention behaviors and selectivity for different hydrophilic analytes [17, 18].

### 1.3 QuEChERS method

As international food trading grows rapidly, food safety screening techniques have become more important. To support this need, QuEChERS sample treatment was first proposed by Anastassiades et al. [19] and has been since widely used in food laboratories. Food samples were pretreated by a combination of acetonitrile liquid-liquid extraction (LLE) and dispersive solid-phase extraction (dSPE) to remove impurities in the matrix. Over the past few years, QuEChERS has been used for the determination of insecticides in fruits [20], vegetables [21], and other kinds of food products [22]. Common sorbents that are used in QuEChERS dSPE include graphitized carbon black (GCB), and C18 bonded silica. The addition of other reagents, such as primary and secondary amines (PSA) or anhydrous magnesium sulfate, can also be included. Each of the components has a function for removal of interferences, such as proteins and pigments [23]. The QuEChERS method greatly simplifies the sample pretreatment procedure, reduces total analysis time, and is effective in reducing matrix interferences. From previous literatures, acceptable precision and accuracy were generally obtained. Belemguer et al. [24] analyzed 40 insecticides in water and fish using the QuEChERS method prior to LC-MS/MS. They reported that the increasing levels of insecticides concentration in Júcar River (eastern Spain) were threatening the local ecosystem. They indicated that extraction recoveries for most insecticides ranged from 70 – 100%, and all matrix effects were lower than 20%. Norli et al. [25] reported organic pollutant residues in

fish at Lake Koka in Ethiopia by applying QuEChERS extraction technique with good recoveries and detection limits. Other related techniques for fish matrices cleanup were also reported [26, 27].

## 1.4 Recent developments in HILIC and QuEChERS

In recent years, there has been a proliferation of research concerned with using the QuEChERS method in the combination with HILIC mode separations. Both of the techniques are being further modified and improved in advanced applications. As mentioned in section 1.3, QuEChERS method is widely applied in the research of food monitoring, especially for the determination of multi-pesticide residues in variety of food sample matrices. The choice of dSPE materials according to properties of sample matrix is one of the crucial factors for recovery of analytes. The selection of different composition of organic solvents or types for sample extraction also plays an important role. Typical QuRChERS method yields its final extract in acetonitrile. Acetonitrile is the most commonly used solvent with QuEChERS method because of its ability to extract a wide range for pesticides, as well as other compounds, while eliminating unwanted compounds present in sample matrix. Acetonitrile is also the most commonly used organic mobile phase component in HILIC-MS, making it more desirable to use as the extraction solvent. There are other possible alternative organic solvents for QuEChERS extraction. For example, acidified methanol was used in a Quick Polar Pesticides (QuPPe) method, which was developed to determine non-QuEChERS-amenable highly polar pesticides [28]. Nguyen et al. [15] pointed out the effect of mobile phases in HILIC mode with comparing acetonitrile with three other solvents. Although methanol can dissolve some chemicals that are not soluble in acetonitrile, the strong hydrogen bonding interactions between mobile phase and stationary phase is a drawback in HILIC and

might cause little or no retention of analytes [29]. Acetonitrile was shown to be a superior solvent for separation, due to its lack of hydrogen bonding with the stationary phase compared to other solvents.

Chung et al. reported the determination of 15 pyrethroid (PYR) pesticides and two metabolites of dithiocarbamates, ethylenethiourea (ETU) and iso-propylenethiourea (i-PTU), in a variety of food samples [30]. For sample extraction, they suggested increase the amount of C18 sorbents in dSPE when the fat content of sample was expected to be greater than 2%. They applied GCB sorbents for treatment of color-intense samples. Gas chromatography (GC)-MS with a fused-silica capillary column gave poor responses of PYRs in electron impact mode, and low sensitivity for some PYRs in LC-MS analysis with a C18 column. ETU and i-PTU were not retained in RPLC, but a HILIC column was introduced to address this problem. ETU and i-PTU were determined under an acidic condition HILIC mode by adding formic acid. Both compounds were well separated from the solvent peak and matrix, meaning better selectivity was achieved. Limits of quantification (LOQ) were 10 and 5 μg/kg for PYRs and i-PTU respectively, and recoveries were greater than 70% in all food samples.

Zhou et al. also reported a rapid determination method for ETU in potato and cucumber [31]. The recovery of ETU in potato was about 30% while using acetonitrile with 1% formic acid as extraction solvent, and 40% when pure acetonitrile was applied. They found that under alkaline extraction conditions (acetonitrile with 1% ammonium hydroxide), ETU recovery could reach to almost 100%. Separations were carried out on a ZIC-pHILIC column. They pointed out that the surface adsorption was the most responsible retention mechanism for ETU by investigating buffer fraction versus capacity factor.

Li et al. [32] developed a rapid method for determination of Chlormequat (CQ) in meat by combining HILIC and QuEChERS techniques. CQ is a widely used plant growth regulator in modern agriculture, and was also reported to accumulate in livestock and poultry meat samples. Previous literature regarding the determination of CQ in animal tissues provided insufficient LOQ to meet the requirement for practical usage. In this literature, PSA, GCB, and C18 sorbent were evaluated for dSPE usage. However, the presence of PSA and GCB gave poor recoveries of CQ (<43.5%); only the recovery using a C18 sorbent alone was within acceptable range. Although significant ion suppressions were found in beef, goat, and pork meat sample from the matrix effect evaluation, excellent limit of detection (LOD) and LOQ were achieved (0.03 and 0.1 μg/kg, respectively), and the LOD was 500 times better than the previously reported method.

The first application of QuEChERS method in trace analysis for paralytic shellfish poisoning (PSP) toxins in seafood was reported by Zhuo et al [33]. They investigated four different PSP and three epimeric pairs of toxins in the study. All PSP toxins were well separated by an amide HILIC column. Shellfish sample matrices were cleaned by hydrophilic lipophilic balanced (HLB) and GCB dSPE sorbents. Ion suppressions of PSP compounds were very strong in all shellfish matrices. To reduce matrix effects from the interferences, they diluted the sample, but this decreased the sensitivity of the method.

Improvements in HILIC techniques and availabilities of different types of commercial HILIC columns has increased the possibility to determine several difficult chemical classes not addressable by RP or NPLC modes. An excellent review has discussed analysis methods of orphan and other difficult pesticides including highly polar compounds and ionic herbicides The author listed many feasible ways that HILIC and

QuEChERS could be combined to address problems with the separation of analytes that could not be done by RPLC and NPLC [34].

Kittlaus et al. [35] proposed a framework for an automated two-dimensional (2-D) LC mass spectrometry (LC-MS) system, combing HILIC and RPLC for matrix removal and analyte separation. More than 300 pesticides were determined in various food commodities (cucumber, lemon, wheat flour, walnut, and rocket). The target analytes and matrix components were separated in the first dimension of LC system. A diol functionalized HILIC column was introduced in the first dimension, primarily for removal of matrix interferences. Retention was posited to be based on the concept of partitioning of sample components between organic and the water phases. It was very similar to QuEChERS liquid-liquid extraction between water and acetonitrile layers due to the lack of charged functional groups on the surface of the diol column. The recoveries of most pesticides analyzed were within an acceptable range from 70% - 120%. In addition, several columns with different functional groups were also evaluated for their suitability. The result showed that YMC-Pack Diol was most suitable for the separation of analytes and matrix compounds. This method provided an alternative way to cleanup food sample and also accelerated the process of pesticide analysis.

Three common sample preparation methods in food are QuEChERS, DFG S19, and ChemElut [36-38]. Each offers different approaches to remove various matrix interferences present in food samples. In the field of sample preparation, matrix effects are the primary problem to be addressed. With the development of automated 2-D LC system in the previous literature, Kittlaus et al. [39] compared these three methods and evaluated the reduction of matrix effects from cucumber, lemon, wheat flour, and black tea. The extractions were carried out with different organic solvents among the methods - acetonitrile in QuEChERS, methanol in ChemElut, and acetone in DFG S19. However,

organic solvents only had minor impacts on matrix effects. Although none of the methods could fully remove all interferences, all three methods were capable of removing highly polar compounds, and their performances varied in different types of sample matrices. QuEChERS method showed more reduction of the ion suppression than ChemElut and DFGS19 in wheat flour, cucumber, and lemon samples. Matrix effect profiles were constructed by LC-MS analysis of final extracts obtained from three different ways of sample pretreatment methods: raw samples that were only extracted with acetonitrile, QuEChERS method, and the newly developed 2-D method. Results of the new method showed fewer matrix effects in all the samples. The developed 2-D HILIC-RP-LC-MS/MS technique was more effective than the three off-line sample preparation methods.

Besides food samples, the combination of HILIC and QuEChERS methods were also applied to the extraction of lipids from blood plasma and urine [40]. Dae et al. noted that lipids are very diverse in their hydrophilic and hydrophobic properties, which makes it even harder to extract all lipid classes with high recoveries at the same time using the traditional Folch extraction method [41]. The QuEChERS method was modified in their research to be used for lipid extraction. They evaluated the recoveries of 19 lipid standards of different classes by varying extraction organic solvents (acetonitrile, methanol, chloroform/methanol, and methyl tert-butyl ether/methanol). The comparison of various dSPE adsorbents including strong anion exchange resin (SAX) sorbents showed that C18 particles indicated relatively good performance in recoveries (>80%) compared to other types of sorbents. The optimized lipid analysis in QuEChERS method was carried out on an unmodified silica HILIC column. For both blood plasma and urine samples, the MS signals of lipids were improved by decreasing ion suppressions compared to the Folch extraction method. Matrix effects obtained from Folch method ranged from 2 to 31% with ion suppression and enhancement in different lipids, but for

QuEChERS case, no ion suppression was observed for all the 19 lipids, and matrix effects were within 17% in both urine and plasma sample. Dae et el. also highlighted the developed high-speed extraction technique could obtain equivalent results for successful identifications of lipids compared to the more time-consuming Folch method.

## 1.5 Aim of the work

The aim of our work was to quantify nicotine and its metabolites in fish tissue, as well as to study the matrix effects resulting from the application of marine versus freshwater fish with QuEChERS method. A HILIC method featuring a bare silica phase coupled with triple-quadrupole electrospray tandem mass spectrometry was used for quantitative determination. Our goal was to develop a fast, easy, and sensitive way for measuring nicotine and metabolites in fish tissue with a low-cost but effective sample preparation method.

# Chapter 2

# Determination of Nicotine and its Metabolites Accumulated in Fish Tissue

## 2.1 Experimental

### *2.1.1 Chemical and materials*

Nicotine was purchased from Fluka; cotinine and anabasine were purchased from Sigma-Aldrich. The internal standard, matrine, was also obtained from Sigma-Aldrich. The alternative internal standard, nicotine-D4, was purchased from Cerilliant. Sodium hydroxide was obtained from EMD Chemicals. Formic acid was obtained from Sigma-Aldrich. Ammonium formate was obtained from Acros Organics. Liquid chromatography-mass spectrometry-grade acetonitrile and water were supplied by Honeywell Burdick and Jackson. Column used for analysis was Agilent Poroshell 120 HILIC Threaded Column (2.1 mm i.d. × 100 mm L, 2.7 µm dp). Frozen fish fillets of salmon and tuna were purchased from Central Market; catfish and tilapia were purchased from Target. All fish fillets were cut into small pieces (< 0.5 $cm^2$) and were homogenized by a commercial grinder. Homogenized samples were kept by a Ziploc plastic bag and stored in a freezer at -20℃ until further use. AOA C QuEChERS packages including extraction tubes, salt pouches containing magnesium sulfate and sodium acetate, ceramic homogenizers, and dSPE tubes were all supplied by Agilent.

### *2.1.2 Preparation of stock solutions, calibration standards, and quality control samples*

Stock solutions of nicotine, cotinine, anabasine, and matrine were prepared by accurately weighing and dissolving approximately 10.0 mg of each compound in 1g of acetonitrile. Nicotine-$D_4$ was 100 µg/mL in acetonitrile. Standard working solutions were prepared at 100 and 1 µg/g by diluting the stock solutions with acetonitrile. Blank fish matrix extracts acquired from the QuEChERS method were evaporated to dryness under

a stream of nitrogen. And the matrix-matched calibration curve samples were prepared in duplicate by adding an appropriate amount of working standard solution to the dried-out matrix residue to obtain matrix-matched standards of 0, 5, 10, 25, 50, 75 and 100 ng/g in acetonitrile. These concentrations could be converted by multiplying each with 7.5. (0, 37.5, 75, 287.5, 375, 562.5, and 750 ng/g in fish.) Quality control samples were prepared in sextuplicate at three concentration levels (75, 300, and 600 ng/g in fish). Internal standard was spiked in all prepared and unknown samples at 10 ng/g in fish, before processing. All solutions were stored in a freezer at 4℃, and standards were kept at -20℃.

*2.1.3 Sample preparation of modified QuEChERS method*

The modified QuEChERS method is outlined in Figure 2-1. [42, 43] 2 g (± 0.1 g) of fish were carefully weighted and were put into a 50 mL AOAC extraction tube. A volume of 13 mL of water was added to make a total loading of 15 g, and internal standard was spiked in. The mixture was vortexed and also pH value was adjusted with 0.5 M NaOH to approximately 11. A volume of 15 mL of acetonitrile was added on. A salt pouch contained 6 g magnesium sulfate and 1.5 g sodium acetate was opened and was poured into the extraction tube accompanied with two ceramic homogenizers. The extraction tube was tightly closed, shaken vigorously for one minute. After centrifugation, 4 distinctive layers appeared. From the bottom to the top were undissolved salt, aqueous layer, fish matrix solid layer, and acetonitrile layer, respectively. 1 mL supernatant was transferred into a dSPE cartridge and followed with 30 seconds of vortexing. The dSPE tube was centrifuged for 5 minutes at 10,00 RPM, and then 0.4 mL extracts was filtered by a 0.22μm PTFE Membrane filter. The filtrate was loaded into an autosampler vial, and then transferred to the autosampler for LC-MS/MS analysis. Completion of the entire

sample preparation is achievable in 40 minutes, and multiple samples can be processed in parallel.

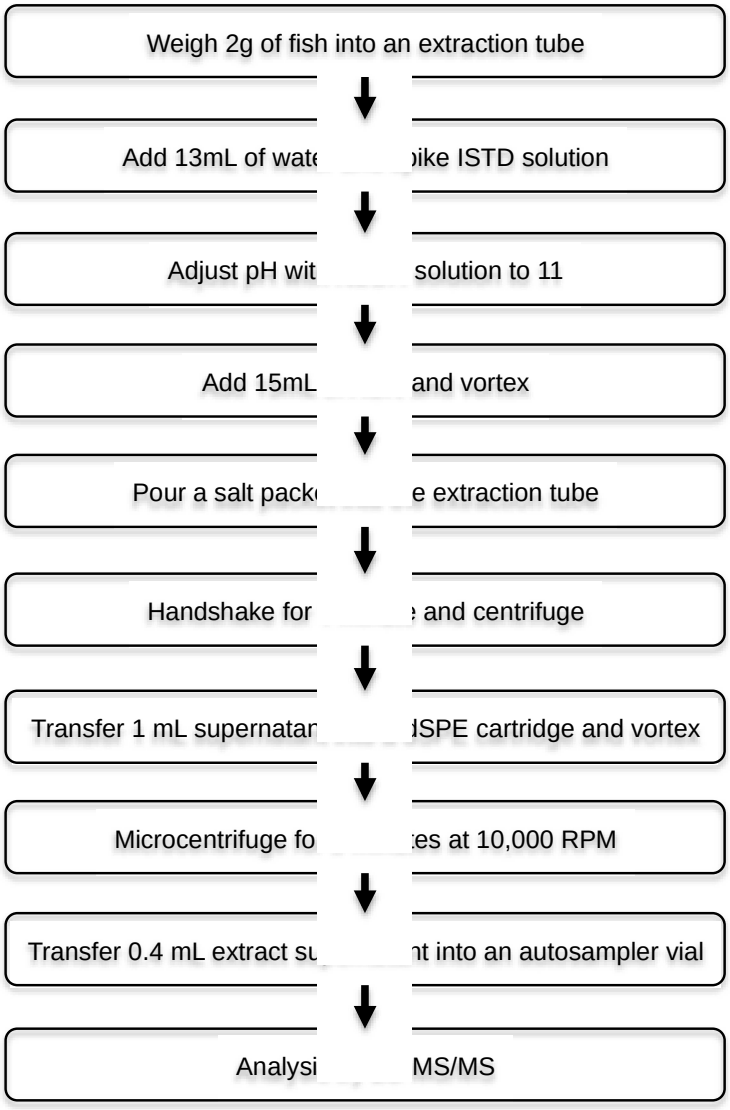


Figure 2-1 An overview of the modified QuEChERS workflow.

ISTD = Internal standard; NaOH = Sodium hydroxide; ACN = acetonitrile

*2.1.4 LOD, recovery, and matrix effect*

Samples used for limit of detection were prepared in septuplicate at a concentration of 0.5 ng/g in acetonitrile (3.75 ng/g in fish). The accuracy and precision were determined by analyzing the quality control samples in sextuplicate, at low, medium, and high concentrations. The recovery was evaluated by comparing the response of the analytes spiked before and after extraction. The relative slopes of calibration curves in matrix-matched standards versus no matrix standards were used to evaluate the presence of matrix effects in the experiment.

*2.1.5 LC-MS/MS conditions*

High-performance liquid chromatography analysis was performed with a Shimadzu Prominence LC system (Shimadzu USA Manufacturing, Inc., Canby, OR, USA) consisting of four LC-20AD-XR pumps, a SIL-20A-XR autosampler with a CTO-20AC column oven with 2-position-6-port switching valve. The mobile-phase compositions used in the binary pump were comprised 10mM ammonium formate buffer (pH 3.0) for mobile-phase A and acetonitrile for mobile-phase B [44]. To make the HILIC column work effectively, the column was equilibrated with 30% water in acetonitrile for an hour before use to create a stable water layer on the silica sorbent. The binary pump was operated at a constant flow rate of 0.4 mL/min. For the gradient separation, the binary pump carried out an elution gradient of mobile-phase B from 90 to 70% in 7 minutes, and then the composition of mobile-phase was held at 70% for 30 seconds. The pump was then held at isocratic flow with 90% mobile-phase B for 4.5 minutes. The total analysis time was 12 minutes [42]. Shimadzu LC-MS-8040 was used for the mass analysis in this study. Electrospray ionization was performed in positive-ionization mode with a spray capillary voltage of 4.5 kV, and the detector voltage was 1.72 kV. The curved desolvation

line (CDL) was heated to 300 ℃ and heat block was heated to 400 ℃. Collision-induced dissociation (CID) gas was set to 230 kPa. Nebulizing gas was introduced at 2.0 L/min, and the drying gas was set to 15.0 L/min. Multiple reaction monitoring (MRM) settings along with analyte properties and structures are shown in Figure 2-2 and Table 2-1. Data analysis was performed using LabSolutions software (version 5.53 SP2, Shimadzu).

Table 2-1 Analyte characteristics

| | Analytes | | | Internal standards | |
|---|---|---|---|---|---|
| | **Nicotine** | **Cotinine** | **Anabasine** | **Matrine** | **Nicotine-D4** |
| **log P** | 0.72 ± 0.26 | -0.23 ± 0.38 | 0.85 ± 0.39 | 1.44 ± 0.58 | 0.72 |
| **pKa** | 8.02 | 8.80 | 11.00 | 7.72 | 8.13 |
| **MRM** | 163.20 → 130.05 | 177.00 → 80.05 | 163.20 → 118.05 | 241.10 → 148.10 | 167.20 → 121.05 |
| **Collision Energy** | 21 | 26 | 23 | 33 | 27 |

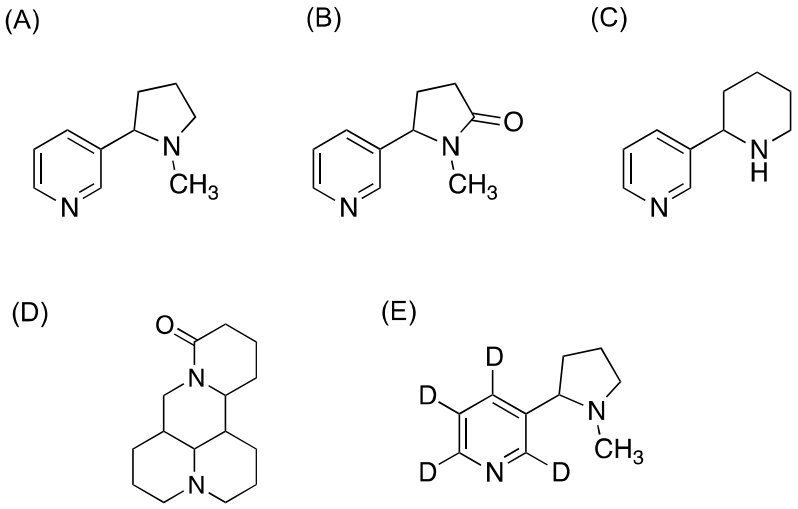


Figure 2-2 Structure of analytes and internal standards

(A) Nicotine (B) Cotinine (C) Anabasine (D) Matrine (E) Nicotine-$D_4$

## 2.2 Results and discussion

### *2.2.1 Analyte separation/ chormatography*

Chromatograms of analytes and their protonated structures are shown in Figure 2-3. All of the peaks are constructed with signals monitoring the transitions of analytes using multiple reaction monitoring. The proposed structures of transitions are shown in Figure 2-4 [45]. Matrine and nicotine-$D_4$ (ISTDs) were retained close to nicotine. The order of elution was cotinine, anabasine, nicotine, nicotine-$D_4$, and then matrine, which was not matched typical HILIC elution order that predicted following the increasing hydrophilicity of analytes. We speculated that other interactions might participate in the separation, such as ion exchange between analytes and stationary phase and electrostatic interactions, causing a more complicated separation mechanism. However, the obtained elution order was observed generally following the order of increasing distribution coefficient (logD). The logD were calculated by equation 2-1, assuming pH value was 3.0 for all analytes, and they were 0.72, -0.24, 0.85, 1.44, and 0.72 for nicotine, cotinine, anabasine, matrine, and nicotine-$D_4$, respectively. In an acidic environment, bi-protonated compound has more hydrophilic properties, which causes more retention in HILIC column. With the assumption, nicotine (pKa1 = 8.02, pKa2 = 3.12), anabasine (pKa1 = 8.77, pKa2 = 4.21), and nicotine-$D_4$ are bi-protonated.

$$logD = logP - \log\left[1 + 10^{(pH-pKa)}\right] \qquad \text{(Equation 2-1)}$$

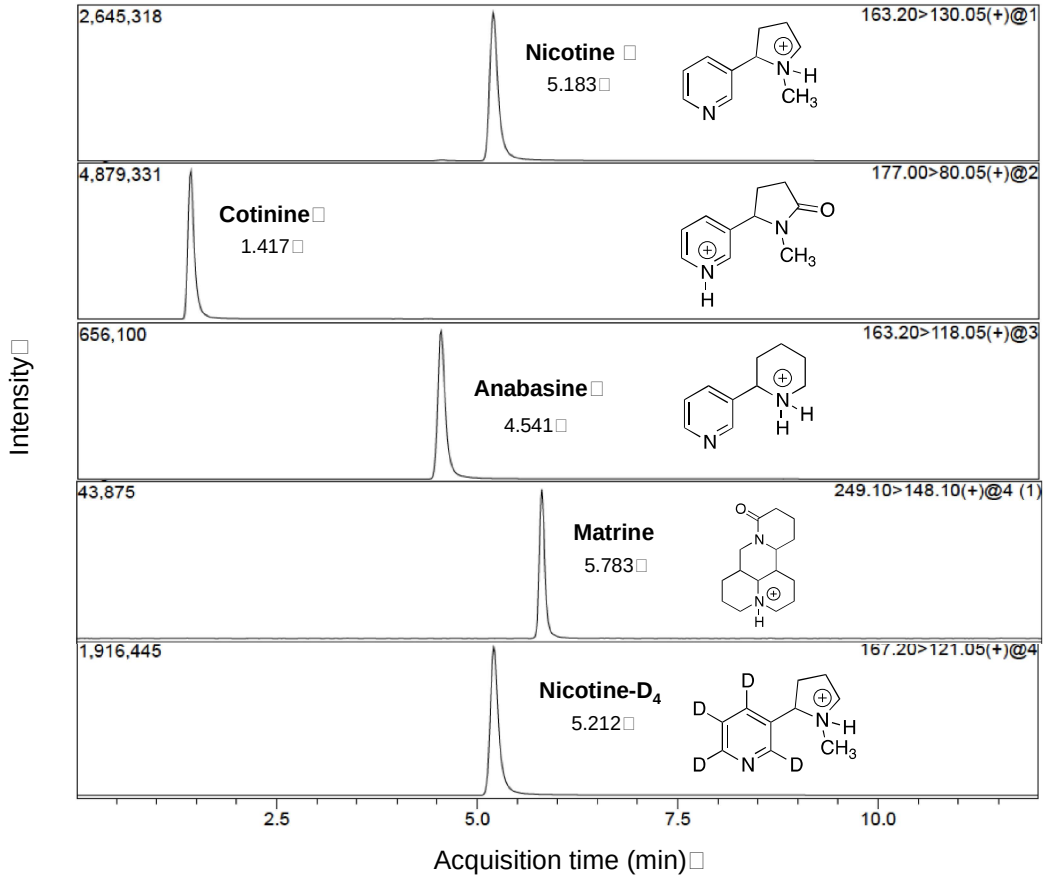


Figure 2-3 Chromatograms of analytes

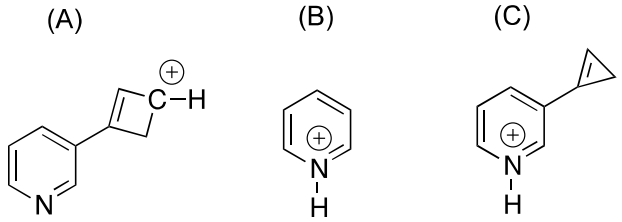


Figure 2-4 Proposed MRM product ion structures of target analytes. [45]

(A) Nicotine: m/z 163➔130 (B) Cotinine: m/z 177➔80 (C) Anabasine: m/z 163➔118

*2.2.2 Variation of QuEChERS dispersive SPE*

Three types of dSPE combinations for sample cleanup were evaluated. Materials in three dSPE cartridges are shown in Table 2-2. PSA has been found to be an effective sorbent for various matrices. It is a weak anion exchange sorbent mainly used for removing organic acids, polar pigments, and sugars through hydrogen bonding interaction [19]. C18 sorbents are used for the removal of lipids and less polar components. End-capped (EC) C18 silica-based sorbents exhibit reduced polar secondary interactions associated with unbonded silanol groups on the surface of the material. This creates better specificity for removal of low polarity interferences [46]. To obtain good recoveries and to reduce matrix effects, applying dSPE for sample cleanup is a critical step. Anhydrous magnesium sulfate is applied for removal of water residues that remained in the organic layer after the LLE step [24].

Table 2-2 Material contents of three dispersive SPE cartridges

| Material | **Fatty samples, AOAC** | **Fruits and Veg, AOAC** | **Drug Residues in Meat** |
|---|---|---|---|
| | Quantity (mg) | Quantity (mg) | Quantity (mg) |
| PSA[a] | 50 | 50 | |
| C18 | | | 25 |
| C18EC | 50 | | |
| Magnesium sulfate | 150 | 150 | 150 |

[a] Primary secondary amine
[b] Endcapped

Blank catfish extracts using different dSPEs were performed separately and spiked respectively with standard solutions at three different concentrations (low, mid, and high; 37.5, 375, and 750 ng/g in fish, respectively), prior to LC-MS analysis. The results are shown in Figure 2-5. The recoveries from the results of dSPE (B), the material suggested for fruits and vegetables were less consistent than those observed for materials (A) and (C). High recoveries (> 150%) of nicotine and cotinine at low concentration indicated that the absence of C18 sorbent may have led to species in the

analyzed samples that caused ion signal enhancement. The results for obtained for dSPE materials (A) and (C) were relatively consistent. However, dSPE (A), designated for fatty samples, which contained both PSA and C18EC sorbents, gave the best extraction recoveries (94 - 103%) of nicotine at low concentrations, and it also gave more consistent and reasonable recoveries of cotinine and anabasine throughout all three concentrations. These results led us to choose dSPE (A) (magnesium sulfate 150 mg, PSA 50 mg, and C18EC 50 mg) as the best interference removal sorbents to use to develop a validated method.

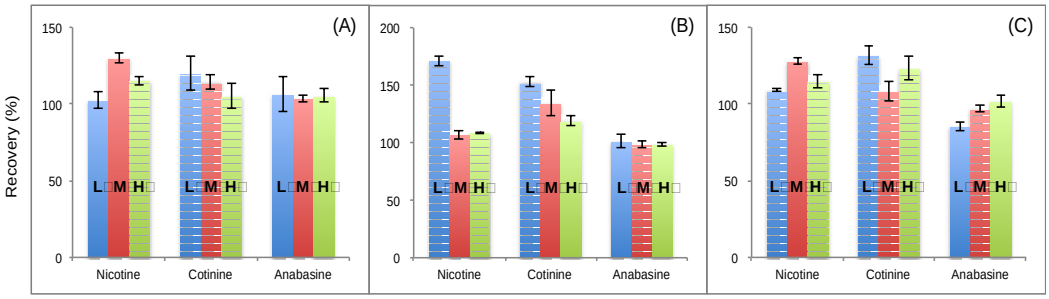


Figure 2-5 Recoveries for different types of dSPE at three concentrations (L = low, M = mid, and H = high): (A) Fatty samples, (B) Fruits and Veg, (C) Drug Residues in Meat.

*2.2.3 Matrix effects*

The main constituents of fish fillet are 60 – 80% of water, 15 – 20% of protein, and a wide range of fats that vary greatly from species to species, and even among individual fish of the same species. Minor components are carbohydrates, minerals, vitamins, sugars, amino acids, and ash [47-49]. Matrix effects from fish tissues could cause inaccuracies of qualitative and quantitative results. Two marine and two freshwater fish samples (tuna and salmon, catfish and tilapia) were chosen to assess the propensity for matrix effects. We compared the results of calibration in fish matrix with those in the

absence of fish matrix to establish regression lines. The experiment was performed three times in all fours samples on separate days. The slopes of the regression lines (deviation from a value of one indicating significant suppression or enhancement of the ion signal in the presence of matrix) are shown in each of the plots in Figure 2-6. In general, matrix effects were minimal. However, significant ion suppression was observed for nicotine in marine fish matrices (10% and 14% reduction in signal, respectively). It was negligible in freshwater fish matrix. No significant matrix effect was observed for cotinine or anabasine either in marine or freshwater fish matrices (≤6%). We speculated that the difference between marine and freshwater matrix revealed on nicotine ion suppression was due to the overall lipid content and fatty acid composition diversities between marine and freshwater fish. Clearly there are significant differences between food sources and habitats for marine vs. freshwater fish [50, 51].

*2.2.4 Method validation*

The method was subjected to strict method validation in conformity with the U.S Food and Drug Administration (FDA) guidelines for bioanalytical method validation [52]. The method was validated for nicotine, cotinine, and anabasine to determine the linearity, accuracy, precision, LOD, LOQ, and extraction recovery for each. Due to the negligible matrix effect observed in freshwater fish, catfish was chosen to perform method validation. Internal standards were used in conjunction with with matrix-matched calibration. Using deuterated nicotine as the internal standard gave slightly better results than using matrine. However, matrine is a viable alternative, and it is cheaper and easier to obtain than the deuterated nicotine standards.

Validation results are given in Table 2-3. The calibration range was established by analyzing standards from 0 to 1500 ng/g in fish for all three compounds. Linearity was

evaluated based on the correlation coefficient ($R^2$) of the best-fit line of the empirical data points. Correlation coefficient in each case was determined that all were greater than 0.995. The accuracy and precision were determined by sampling three different concentrations (75, 300, 600 ng/g in fish) into replicates of six. These concentrations were chosen to represent a low, medium, and high region of the calibration curve. Overall, the precision was higher than 91% for all three compounds and the accuracy was satisfactory at medium and high concentrations. In terms of the poor accuracies at low concentration, we surmised that the co-eluting interferences from the matrix slightly suppressed the ionization efficiency, especially for the early-eluted compound, cotinine. It is well know that matrix effects in electrospray ionization – mass spectrometry can be concentration dependent. The LOD and LOQ were determined by seven replicates at a low concentration along with a matrix-matched calibration curve containing seven points and was then calculated from the equations: LOD = 3s/m, and LOQ = 10s/m, where s was standard deviation of the signal obtained from seven replicates, and m was the slope of the calibration curve. The detection limits for nicotine, cotinine, and anabasine were 9.4, 3.0, 1.4 ng/g in fish, respectively. Extraction recoveries from 61 to 111% were determined by comparing the response signal before and after the extraction at low, medium, and high concentration level quality control samples (see Table 2-3). Acceptable extraction recoveries (70 - 120%) of all three compounds were achieved except anabasine at low concentration (only 61%). Because the associated precision of anabasine at the low concentration was less than 20%, which demonstrated a good precision, a mean recovery below 70% is acceptable, since it is reproducible [53].

Table 2-3 Summary of results of LOD, LOQ, linearity, accuracy error, extraction recovery, and relative standard deviation

| Compound | LOD (ng/g) | LOQ (ng/g) | Linearity, $R^2$ | 75 ng/g (low) | | | 300 ng/g (mid) | | | 600 ng/g (high) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Accuracy error (%) | Extraction Recovery (%) | RSD (%) | Accuracy error (%) | Extraction Recovery (%) | RSD (%) | Accuracy error (%) | Extraction Recovery (%) | RSD (%) |
| Nicotine | 9.4 | 31.2 | 0.998 | -27.9 | 78.3 | 2.4 | +2.8 | 97.5 | 8.7 | +7.8 | 98.1 | 3.0 |
| Cotinine | 3.0 | 9.9 | 0.995 | -46.9 | 77.0 | 7.2 | +1.3 | 111.1 | 3.2 | +3.1 | 111.2 | 5.9 |
| Anabasine | 1.4 | 4.8 | 0.998 | -25.0 | 60.9 | 7.3 | -1.0 | 94.7 | 7.0 | -13.2 | 79.7 | 5.0 |

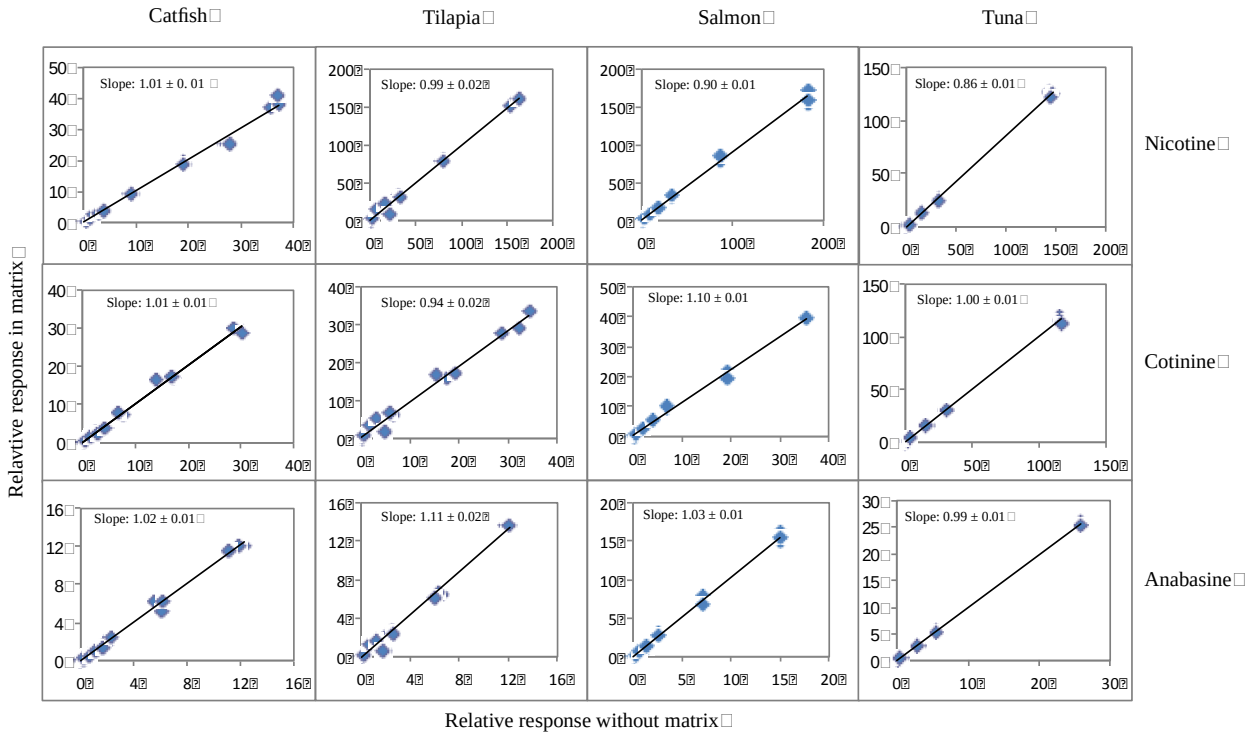


Figure 2-6 Evaluation of matrix effects

*2.2.5 Application*

To demonstrate its practical use, the developed QuEChERS and HILIC method was applied to determine nicotine and its metabolites in 10 fish samples purchased from different local grocery stores. All sample were treated by the method described in section 2.1.3 and analyzed by LC-MS/MS. Cotinine and anabasine were not detected in any of the samples. Two fish samples were shown to have the presence of nicotine but the concentrations were below the LOQ of the method.

The method was also applied to fathead minnow samples provided by Baylor University. Fifteen minnows were exposed in a nicotine-dosed water environment for different periods of time. Three minnows were used as the control group and were kept free from nicotine for 24 hours. The results and details are shown in Table 2-4, as well as the corresponding nicotine exposure water grabbed at each period of time. Nicotine was detected in 12 fathead minnow samples (samples 4-15) and the concentrations in the fish tissue gradually increased from 0.1 to 12 hours of exposure. Cotinine was found in the samples with longer duration of exposure (≥12 hours, samples 10-15). As expected the concentrations varied slightly among individuals, but the results were consistent with expectations and shows good proof-of-principle for the combined QuEChERS and HILIC method.

Table 2-4 Investigation results of fathead minnows and water samples

| Fathead minnows | | | | |
|---|---|---|---|---|
| No. | Exposure time (HR) | Nicotine | Cotinine | Anabasine |
| 1 | 0 | _ | _ | _ |
| 2 | | _ | _ | _ |
| 3 | | _ | _ | _ |
| 4 | 0.1 | 0.47 | _ | _ |
| 5 | | 0.50 | _ | _ |
| 6 | | 0.59 | _ | _ |
| 7 | 1 | 0.97 | _ | _ |
| 8 | | 0.93 | _ | _ |
| 9 | | 0.86 | _ | _ |
| 10 | 12 | 3.68 | 0.06 | _ |
| 11 | | 4.05 | 0.24 | _ |
| 12 | | 4.23 | 0.46 | _ |
| 13 | 24 | 2.39 | 0.04 | _ |
| 14 | | 3.51 | 0.31 | _ |
| 15 | | 3.03 | 0.12 | _ |
| | | | | (μg/g in fish) |
| Water | | | | |
| 1 | 0.1 | 1.68 | _ | _ |
| 2 | 1 | 3.86 | _ | _ |
| 3 | 12 | 5.22 | _ | _ |
| _: None detected | | | | (μg/g) |

# Chapter 3

## Summary and Future work

In this study, a QuEChERS sample preparation protocol involving liquid-liquid extraction with acetonitrile and dSPE was shown to provide for analytical determination of nicotine and its metabolites using LC-MS/MS with minimal or no matrix effects. Depending on the type of matrices, the combinations of the materials in dSPE cartridge can be changed for different sample types to improve the performance. With HILIC mode separation, all compounds were separated and with good specificity and satisfactory recovery data. Moreover, the final solvent composition containing the analytes after extraction is highly compatible with the HILIC separation mode. Nicotine maximum residue limit (MRL) were set at 0.01 mg/kg for all commodities [54]. And the acceptable daily intake (ADI) was 0.0008 mg/kg body weight [55]. The LOD and LOQ of nicotine of the developed method were below MRL levels and are feasible for nicotine residues monitoring. For most of the fish samples colleted from grocery stores were free from nicotine contamination. Despite some fish products were contaminated by nicotine, the concentration were below MRL level, indicating there is no harm for human consumption. In ongoing studies, this developed method would be evaluated by determination of contaminants on other seafood products. And also, several different types of dispersive SPE materials applied in sample extration would be further investigated to better adapt in various aquatic organism samples according to their characteristics.

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## Biographical Information

Yun-Wei Chang was born in Hsinchu, Taiwan, in 1987. He grew up and stayed in Hsinchu until finished high school at the age of 18. He then attended the Chung Cheng National University in Chiayi, where he obtained his Bachelor's of Science in Chemistry and Biochemistry. After graduation in 2009 spring, he joined Taiwan National Military Service. He served for one year in Military Police leadership position as Corporal at Office of the President, Taipei, Taiwan. Since August 2010, he worked for UEI JONG JANG CO., LTD. firm for two years after the year of military serveice and was in a position of Process Engineer and Manager. And then he enrolled in a Master program in University of Texas at Arlington in 2012 Fall. Starting the winter of junior year in university, he participated in a research project of determination and optimization on traditional Chinese medicine (curcumin, genipin) using LC-MS coupled with Taguchi orthogornal array techniques, which was under the supervising of Dr. Shau-Chun Wang. At the end of first semster in University of Texas at Arlington, he focued on analytical chemistry and studied under the guidance of Dr. Kevin A. Schug. During the time he worked in Dr. Schug's lab, he explored many area of chemistry and acquired fundamental knowledge of analytical techniques. He also gained a lot of separation experience by participating in a project of HILIC mode separation using QuEChERS sample extraction technique coupled with LC-MS.