INVESTIGATION OF ARSENIC ACCUMULATION IN DEEP-FRYING OIL

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Li Zhen Yan

Abstract

Deep-frying is a popularly used cooking method, and the deterioration of deep-frying oil has been a topic of concern and has been extensively studied. However, the transfer and accumulation of heavy metals from the food being deep-fried to the deep-frying oil has not been well studied although it has been proven possible and poses high risk due to their highly toxic nature. This study aims to address this issue by a series of deep-frying experiments by repeatedly using rapeseed oil, a commonly used frying oil, and settings that mimicked the typical food trade practice.

Oyster was the first type of food to be tested due to its being commonly deep-fried and high heavy metal levels. The results showed that arsenic could accumulate to over 0.1 ppm, a limit established by the Hong Kong government in edible oil, after 16 deep-frying cycles and could reach as high as over 0.5 ppm with more frying cycles. Further study revealed that the residues in the frying oil contained high concentrations of arsenic (could reach over 6 ppm) and were the major cause of high arsenic concentrations of the deep-frying oil. It was found that the residues were formed from liquid leached from the oysters during the deep-frying. The study also demonstrated that arsenic could be transferred from the deep-frying oil to the food being deep-fried by adherence of the residues onto the food.

Deep-frying of grouper fish fillets yielded deep-frying oil with arsenic concentrations similar to that of the oysters, while the colours of the oil and the residues were significantly lighter compared to those of the oysters. The lighter colour and the slower deterioration rate of the deep-frying oil would likely lead to delayed replacement of oil, leading to higher food safety risks as oil with high arsenic content would not be replaced in time. In addition, the results

demonstrated that heavy metal accumulation in deep-frying oil was not limited to oysters but also other types of deep-fried food, leading to wider implications.

Arsenic speciation analysis was performed to evaluate the potential food risks associated with consumption of deep-frying oil and deep-fried food of high arsenic levels. The preliminary results showed the potential food safety concerns since the levels of dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA), both carcinogenic, increased in the deep-fried food, the residues and the deep-frying oil, and deep-frying might cause demethylation of organic arsenic species, transforming them into more toxic forms. Further studies are needed to completely understand the conversion of the arsenic species during deep-frying.

In summary, our results revealed that arsenic could be accumulated to very high concentrations in repeatedly used deep-frying oil when frying foods such as oysters and grouper fish fillets, posing risk to the consumers. Replacing deep-frying oil based on oil colours and deterioration may not be suitable in such cases, and new guidelines and quality indicators for heavy metal contaminations in deep-frying oil should be considered. Our study also indicated that removing residues from the frying oil and using batter to mask the food before the frying could help to reduce accumulation of heavy metals in deep-frying oil, which should also be recommended to the food trade.

Research Publications

Conference Papers

1. **Li, Z. Y.**, Yao Z. P. Accumulation of Arsenic in Deep-frying Oil: An Investigation with ICP-MS, Hong Kong Society of Mass Spectrometry Symposium 2022, Hong Kong, 11 June 2022. (Outstanding Oral Presentation Award)
2. **Li, Z. Y.**, Yao Z. P. Investigation of Arsenic Accumulation in Deep-frying Oil, 29th Symposium on Chemistry Postgraduate Research in Hong Kong, Hong Kong, 12 November 2022.
3. **Li, Z. Y.**, Yao Z. P. Investigation of Arsenic Accumulation in Deep-frying Oil, 71st American Society of Mass Spectrometry Conference, Houston, 4-8 June 2023.
4. **Li, Z. Y.**, Yao Z. P. Investigation of Arsenic Accumulation in Deep-frying Oil, Hong Kong Society of Mass Spectrometry Symposium 2023, Hong Kong, 17 June 2023.

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List of Abbreviations

Full Form	Abbreviation
Total polar compounds	TPC
Monomethylarsonate	MMA
Dimethylarsinate	DMA
Arsenobetaine	AsB
Arsenocholine	AsC
Trimethylarsine oxide	TMAO
Tetramethylarsonium	TMA^{+}
Inorganic arsenic	iAs
Dimethylmonothioarsinic acid	DMMTA
Parts per million	ppm
Parts per billion	ppb
Scanning electron microscopy	SEM
Energy dispersive X-ray spectroscopy	EDX
Liquid chromatography	LC
High-performance liquid chromatography	HPLC
Inductively coupled plasma	ICP
Electrospray ionization	ESI
Mass spectrometry	MS
Quadrupole-Time-of-Flight Mass Spectrometer	QTOF
mass-to-charge ratio	*m/z*
Centre for Food Safety	CFS
Roxarsone	ROX

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Chapter 1:Introduction

1.1. Introduction to deep frying

Deep-frying, or sometimes referred to as deep fat frying, is a method of cooking by submerging food in hot fat or oil. Deep-frying is widely used by the food industry due to the popularity of its unique texture and fragrance amongst consumers[1] and profit margins of deep-fried food being as high as 75%[2]. Repeated use of deep-frying oil is typical in commercial practice for cost efficiency, but the oil can deteriorate over time, affecting deep-fried food quality (Figure 1-1) and more importantly increasing the health risk associated with consumption[3].

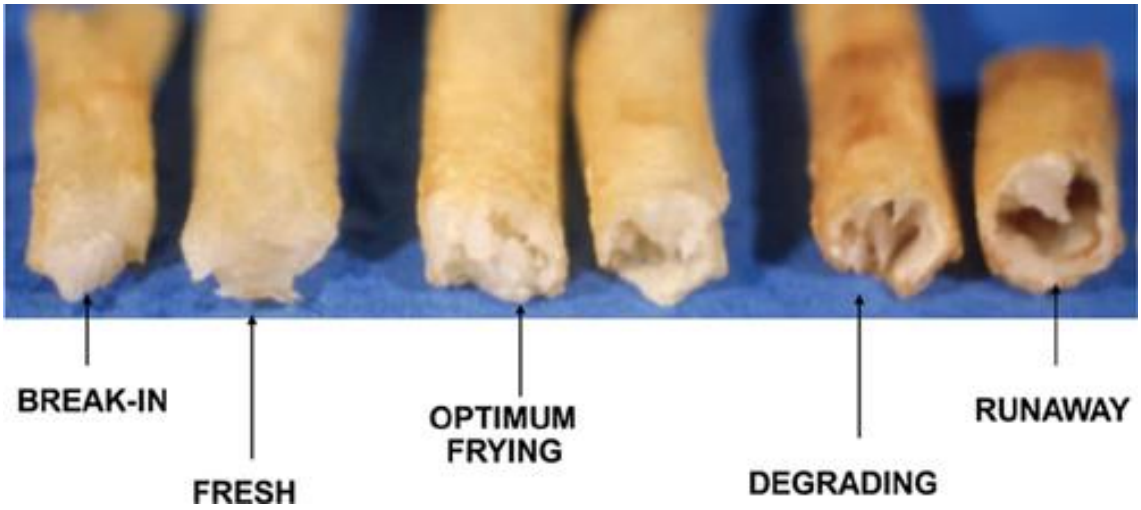


Figure 1-1: Potato chips deep-fried in progressively deteriorated deep-frying oils[4]. It is believed that deep-fried food with best quality is produced when the oil is "broken-in", in which the oil contains more surfactants from deterioration reactions to allow food to be properly cooked.

Deep-frying oil may deteriorate due to several factors. The most studied and discussed routes of deterioration are oxidation, hydrolysis, and polymerization reactions (Figure 1-

2) between the deep-frying oil and oxygen or moisture under elevated temperatures[4]. Deep-frying oils are often heated to 150-180 °C during deep-frying operations, and triglycerides, the main component of edible oil, can react with the moisture from the food being deep-fried and atmospheric oxygen to give unwanted products[4-6]. Other possible reactions such as starch gelatinization, protein denaturation browning and Maillard reaction can also occur but mostly within the food being deep-fried[3]. Reported health risks associated with the consumption of deteriorated deep-frying oil include acute adverse effects such as gastrointestinal distress and chronic health effects such as hypertension, cardiovascular diseases, atherosclerosis and cancer[7].

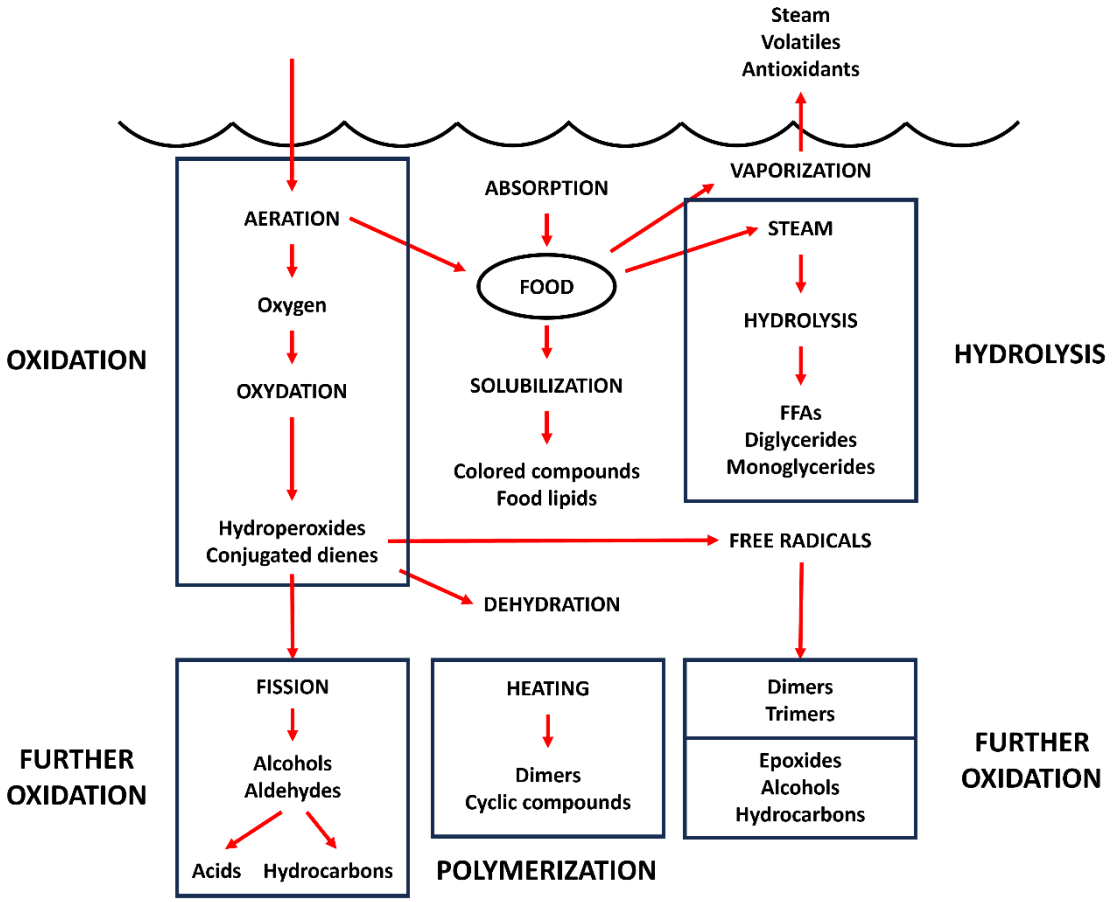


Figure 1-2: Summary of deterioration reactions[4]. These reactions, reported in the 1980s but remains valid, are categorized into oxidation, hydrolysis, or polymerization reactions.

Measuring the deep-frying oil quality is therefore critical to preventing oil abuse and ensuring deep-fried food safety. However, not all deterioration reactions and their products have been fully elucidated despite extensive studies. The innumerable combinations of oil type and food type together with various other contributing factors, such as turnover rate, oil temperature, amount of available moisture and oxygen, and amount of food debris to name a few, cause the deterioration reactions to be extremely complex[6, 8, 9]. Thus, instead of focusing on levels of specific compounds that may vary greatly from operation to operation, researchers have employed quality indicators for the evaluation of deep-frying oil quality. Examples of quality indicators include organoleptic indicators such as colour, smell, foaming and viscosity, and chemical indicators such as free fatty acid content and polymer content[1]. Of all quality indicators, total polar compounds (TPC), is one of the most well-rounded quality indicators as the measurement of TPC includes most deterioration reaction products such as free fatty acids, glycerol, mono- and diglycerides, aldehydes, ketones etc. Thus, TPC is the most used quality indicator in regulations and recommendations by regulatory bodies and organizations especially in recent years[1, 10, 11].

1.2. Accumulation risk of contaminants in deep-frying oil

Compared to the extensive studies on buildup of deep-frying oil deterioration by-products[12-17], there are few studies on contaminants transferred from the food being deep-fried to the deep-frying oil. It has been demonstrated that there is mass transfer from food to oil during frying, most notably lipids including phospholipids, cholesterol, pigments, and lipid-soluble content including Maillard browning products, phenolic compounds, lipid-soluble vitamins and lastly trace metals[6, 18]. Transfer of content from food to deep-frying oil can be problematic as the content can be incorporated in deep-fried food during subsequent deep-frying operations, and can pose food safety issue if said content is harmful. Lehrer et al. [19] demonstrated that shrimp allergens can transfer from shrimps being fried to the deep-frying oil, and transferred to subsequent batches of food deep-fried. Although the concentration was unlikely to cause clinically significant allergic reactions under the experimental circumstances, unintended and unwanted exposure was proved possible.

Among the abovementioned possible contaminants, trace metal contaminants raise a high level of concern. A study by Artz et al. [20] investigated the accumulation of iron in deep-frying oil and found that deep-frying meat can lead to increase in iron content of repeatedly used deep-frying oil, demonstrating that accumulation of heavy metals in deep-frying oil is possible. The threshold for toxic effects is relatively high at 20mg/kg[21], and thus pose a less significant health risk even if it accumulates in the deep-frying oil. However, for other heavy metals such as arsenic and lead, the health risk associated can be severe at low levels of exposure. Therefore, the accumulation of these metal in deep-frying oil, if any, can be a significant food safety concern. Atia et al. [22] also revealed

possible transfer of heavy metals from food to cooking oil, as they observed a reduction of 60%, 40%, 30%, and 20% for mercury, lead, cadmium, and arsenic respectively when pan-frying shellfish. They proposed that "reduction in pan-frying might be attributed to dissolving of the metals in the cooking oil", which is to some extent consistent with the observation made by Artz et al. Lastly, a study by Chang & Jiang[23] demonstrated the possibility of increase in arsenic and cadmium levels in cooking oil after frying. It was found that the arsenic and cadmium concentrations in soybean oil were 0.13 and 0.23 $\mu g/g^{-1}$ before frying, and were 1.98 and 0.78 $\mu g/g^{-1}$ after frying respectively. Chang & Jiang did not describe their method of frying in detail, but the possibility of accumulation was demonstrated nonetheless.

Evidence in the literature suggested that accumulation of heavy metals is possible in cooking oil during frying or deep-frying. Out of the mentioned studies, the heavy metals Atia et al. and Chang & Jiang investigated poses much more significant health risks if consumed, and thus will be further discussed.

1.3. Health risk associated with heavy metals

In earlier years, "heavy metals" is often used in literature and legislation as a group name to describe metals and metalloids that have been linked to contamination and toxicity[24] and the use of the term had been advised against by some[24] in recent years due to the ambiguous nature and inconsistent use of the term. Regardless of how they are named, the consensus is that these persistent contaminants have long been a concern in food safety due to their persistence in terms of both biodegradability and thermal stability, leading to a tendency for them to accumulate. Even if the initial concentrations of these

contaminants were deemed harmless, the levels can ultimately become significant and cause adverse health effects.

Food products may contain varying amounts of heavy metals, and in general the concentrations of these metals are highest in seafood products. Total diet studies have shown that seafood products contribute greatly to the intake of heavy metals[25], most notably for arsenic[26]. Thus, for the purpose of this study, focus will be placed on seafood products when studying possible accumulation of heavy metals in deep-frying oil.

1.3.1. Arsenic

Arsenic (As) is an abundant naturally occurring metalloid most well-known for its use as a poison[27], exemplifying its level of toxicity. Arsenic can be found in multiple oxidation states, including As^{0}, As^{3+}, As^{5+}, and As^{3-}, and most commonly as As^{3+} and As^{5+}. The study of arsenic species, or arsenicals, is a lot more complex compared to the study of species of most metallic elements due to the large number of arsenicals identified[28] (often categorized as lipid-soluble or water-soluble[4]) (Table S-1).

Due to the varying toxicities of arsenic species, many studies have focused their effort on arsenic speciation from various sources. It was found that drinking water and terrestrial organisms primarily contained inorganic arsenic[29, 30], and marine organisms primarily contained organic arsenic species[31, 32]. Common arsenic species found in marine organisms include monomethylarsonate (MMA) and dimethylarsinate (DMA), and to a lesser extent arsenobetaine (AsB), arsenocholine (AsC), trimethylarsine oxide (TMAO) and tetramethylarsonium ion (TMA^{+}). The transformation of inorganic arsenic to organic

arsenic species in the marine environment is believed to be the result of biomethylation as a detoxification mechanism in the past decades[31], even though methylation in some cases does not reduce the toxicity.

Degrees of toxicity of arsenicals depend on the species. Albeit a few exceptions, the degree of toxicity of arsenicals generally follows a few rules[28]: trivalent arsenicals are typically more toxic than pentavalent arsenicals; inorganic species are typically more toxic than organic species (one notable exception is that the trivalent organic species can be more toxic than trivalent inorganic species, and therefore methylation would result in an increase in toxicity[28]); and toxicity typically decrease with increasing degree of methylation. Toxicity can vary substantially: Inorganic species can induce both chronic and acute health conditions; meanwhile some organic species, such as AB and AC, are considered non-toxic. Research regarding toxicities of arsenicals, or arsenic speciation in general (Table S1), is still ongoing, as the toxicity of common classes of compounds such as arsenosugars or thioarsenicals has not been fully established in the literature yet[21]. It should also be noted that there are studies[33-35] showing that the absorption of arsenic in the gastrointestinal tracts, or bioaccessability, can be affected by the arsenic species. In a study by Ichikawa et al[33], a type of edible algae is fed to mice and faeces and urine samples were obtained to evaluate arsenic metabolites after consumption. It was shown that the faeces samples contained higher levels of arsenosugars than the urine samples, suggesting that the absorption of arsenosugars is less efficient compared with other arsenicals.

The source of inorganic arsenic is most commonly exposed to human through drinking water consumption. Long-term exposure to high concentrations of inorganic arsenic can

lead to various health risks such as cancers and cardiovascular diseases[36]. The predominant forms of arsenic in drinking water are arsenite, trivalent inorganic arsenic (iAsIII), or arsenate, pentavalent inorganic arsenic (iAsV).

The major mechanism of arsenite toxicity is proposed to be based on the ability for arsenite to readily react with thiol-containing molecules including cysteine and glutathione[37, 38], like in the case of methylmercury, and in turn inhibiting proteins and disrupting intracellular and intercellular processes. For example, arsenite have been demonstrated to be able to inhibit pyruvate dehydrogenase, likely by binding to the lipoic acid functional group of the enzyme. However, it should be noted that Aposhian et al[39] proposed that the binding of arsenite to nonessential sites of proteins may act as a detoxification mechanism.

Organic species, to compare, are predominant in animal food sources. Once thought to be non-toxic[40], some organic species have been demonstrated to be more toxic than their respective inorganic species. It was mentioned earlier that trivalent organic species can be more toxic than trivalent inorganic species, and one prime example is the methylated trivalent arsenic species monomethylarsonous acid (MMA^{III}). MMA^{III}, a methylated trivalent arsenical, was demonstrated to be more toxic then arsenite *in vitro* and *in vivo*[41, 42], and was a more potent inhibitor of pyruvate dehydrogenase[43], suggesting it may induce more severe adverse health effects.

In contrast with trivalent arsenicals, pentavalent organic species generally have decreasing toxicities with increasing level of methylation. Methylated metabolites such as methylarsonic acid (MMA^{V}) and dimethylarsinic acid (DMA^{V}) (Figure 1-3) are

thought to be products of detoxification. Despite that they are still considered to be carcinogenic, the degrees of toxicity and carcinogenicity are reduced with increasing degree of methylation. However, this traditional notion was challenged by studies on the toxicity of dimethylmonothioarsinic acid ($DMMTA^V$). $DMMTA^V$ was demonstrated to be highly reactive with thiol compounds, with a reactivity comparable to MMA^{III} [44], a toxic trivalent arsenical mentioned previously. The toxicity was weaker than MMA^{III} but still higher than other pentavalent arsenicals including monomethylarsonic acid (MMA^V) and dimethylarsinic acid (DMA^V) [45]. Whether there are other exceptions besides $DMMTA^V$ remains unknown. Table 1-1 is a summary of acute toxicity of arsenic in *in vivo* studies.

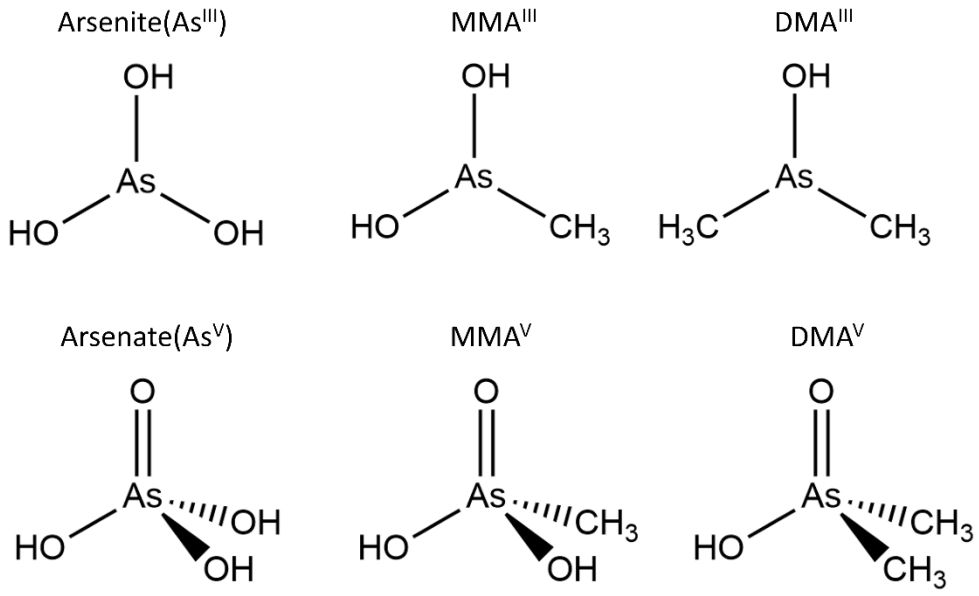


Figure 1-3: Structure of common arsenicals[46].

Table 1-1. Acute toxicity of arsenic in laboratory animals[37].

Chemical	Species	Route	LD_{50} (mg As/kg)	Reference
Arsenic trioxide	Mouse (m)	Oral	26	Kaise et al. [47]
Arsenic trioxide	Mouse (m)	Oral	26-48	Harrisson et al. [48]
Arsenic trioxide	Rat (m/f)	Oral	15	Harrisson et al. [48]
Arsenite	Mouse (m)	im	8	Bencko et al. [49]
Arsenite	Hamster (m)	ip	8	Petrick et al. [43]
Arsenate	Mouse (m)	im	22	Bencko et al. [49]
MMA^{III}	Hamster (m)	ip	2	Petrick et al. [43]
MMAV	Mouse (m)	Oral	916	Kaise et al. [50]
DMAV	Mouse (m)	Oral	648	Kaise et al. [50]
TMAOV	Mouse (m)	Oral	5500	Kaise et al. [50]
Arsenobetaine	Mouse (m)	Oral	>4260	Kaise et al. [47]

1.3.2. Lead

Lead (Pb) poisoning can take the form of acute poisoning, causing acute abdominal pain and pain and weakness in the muscles but is now a rare occurrence in developed countries[51]. Nowadays concerns regarding lead poisoning are in general directed towards chronic exposure to lead, where the severity can range from allergies and weight loss to brain damage and even death[52].

Although there are multiple possible routes of exposure for lead such as the inhalation of airborne particles, ingestion remains to be the most likely route. Contamination of groundwater resources and use of lead containing fertilizers and pesticides all contribute to the accumulation of lead in plants[53], and contamination of seawater and bioaccumulation and biomagnification contribute to the accumulation of lead in seafood[54]. Food originating from plants is considered to be the main source of human dietary lead intake[55], but the intake of lead through seafood can be significant as well. Of various

seafood types, fish generally are not considered a major dietary source of lead, as lead typically accumulates in the livers and kidneys which is often discarded as offal[55]. To compare, lead accumulation in edible parts of molluscs poses more significant food safety concerns. Bioaccumulation of metallic and metalloid contaminants have been reported to be substantial, to the extent where for marine environmental pollution studies bivalve species (i.e. oysters, mussels, clams etc.) are considered biological indicator organisms to monitor metallic pollutants[56]. In general, lead in seafood poses moderate food safety risks. In terms of toxicity, lead can cause various health conditions through a few different mechanisms even at lower concentrations. Lead is absorbed into the bloodstream once inhaled or ingested, transported to other parts of the body, and is thought to accumulate at three destinations – blood, bones and soft tissues[52].

Deposition of lead in bones is notable, as the half-life is estimated to be 20 to 30 years[52]. This is likely because lead ion and calcium ion have the same charge and are similar in size, making the replacement of calcium possible[55]. To compare, the deposition of lead in soft tissues poses even larger concerns despite a much shorter estimated half-life of 40 days; yet it can still cause substantial damage to systems that are primarily composed of soft tissues, the central nervous system and renal system in particular[52]. One of the underlying mechanisms for its neurotoxicity is believed to be its ability to inhibit N-methyl-D-aspartate receptors, which are instrumental in cognitive functions and synaptic plasticity. Eventually this can lead to the disruption of the memory acquisition region of hippocampus, and in turn impair intake and learning skills permanently[57]. Lead can also induce oxidative stress by direct actions on cell membrane and reactions with antioxidants, which can be linked to neurological disorders such as Parkinson's Disease[58]. Effects of lead poisoning in children are also devastating, as lead can cause developmental issues

such as retardation of bone and muscle growth[59, 60] as well as and intellectual development[61, 62], and causing behavioural abnormalities and learning disabilities[63, 64] etc. A summary of the toxic effects in adults and children is shown in Figure 1-4, demonstrating the catastrophic effects lead has on the human body.

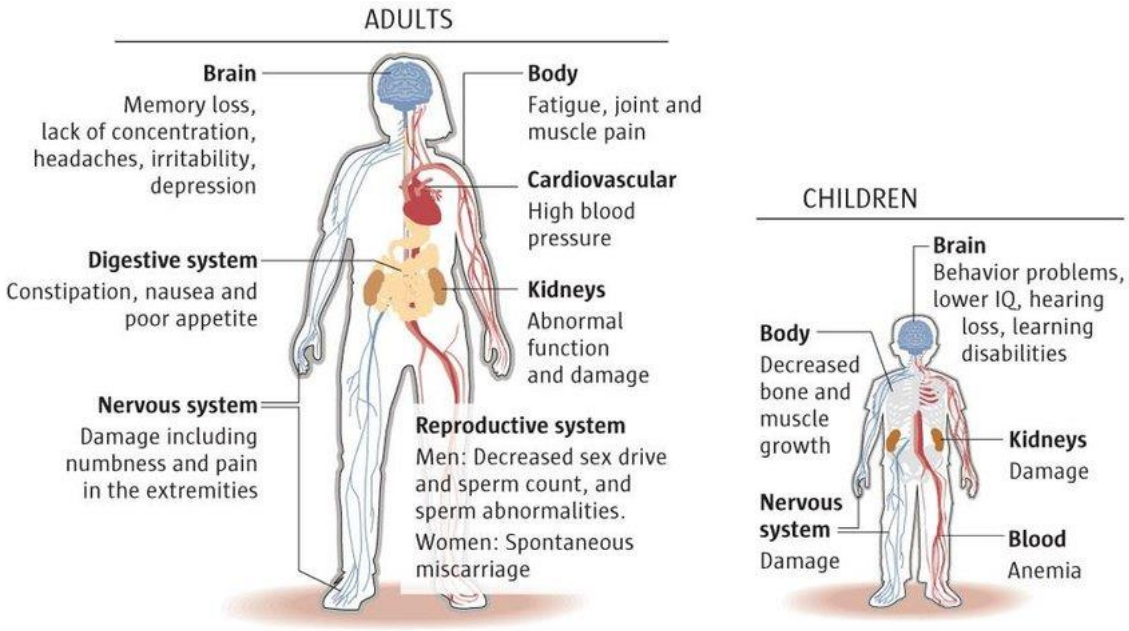


Figure 1-4: Effect of lead poisoning in adults and children[65].

1.3.3. Other highly toxic heavy metals

A few other heavy metals are widely regarded to be of high food safety risks, most notably mercury, cadmium, and chromium, as these three together with arsenic and lead are the top five heavy metals that most commonly induced human poisoning[66]. These elements and their associated toxic effects will be briefly introduced.

Mercury can exist as several forms: Hg^0, Hg^I, Hg^{II}, and Hg-organic, and the most abundant forms in the environment are Hg^0, Hg^{2+}, monomethylmercury, dimethylmercury, and monoethylmercury. The toxicities can vary for different mercury species. Vegetable samples have commonly been found to contain trace amounts of mercury, but methylmercury from fish and other seafood are considered to constitute to over 75% of human exposure to mercury[55].

In contrast with lead which tend to accumulate in the kidneys and livers of fish, mercury can accumulate to higher amounts in the muscle tissues of predatory fish, most notably in tuna[67] both in the white muscles and red muscles[68]. Since muscle tissues are considered edible parts of the fish and are likely to be consumed by human, there is considerable risks of mercury exposure associated with the intake of fish products. Accumulation of methylmercury in molluscs is also pronounced, and although it is proposed that molluscs present less risks of methylmercury exposure to humans compared to predatory fish[69], there have been reports of moderate methylmercury content in edible molluscs in various marine environments[69-71].

Mercury is considered as one of the most toxic metals as it can induce chronic and acute conditions even at low levels of exposure (Figure 1-5). Of the forms it can take, alkyl mercury compounds (in particular methylmercury) are very common and far more toxic than the inorganic form because it can be transferred from blood to the central nervous system through the blood-brain barrier by the large neutral amino acid transporters[53]. Being highly neurotoxic and the predominant form of mercury exposed to human (90% of total absorbed mercury is methylmercury absorbed via the gastrointestinal tract[55]), methylmercury is considered one of the most dangerous environmental pollutants[53].

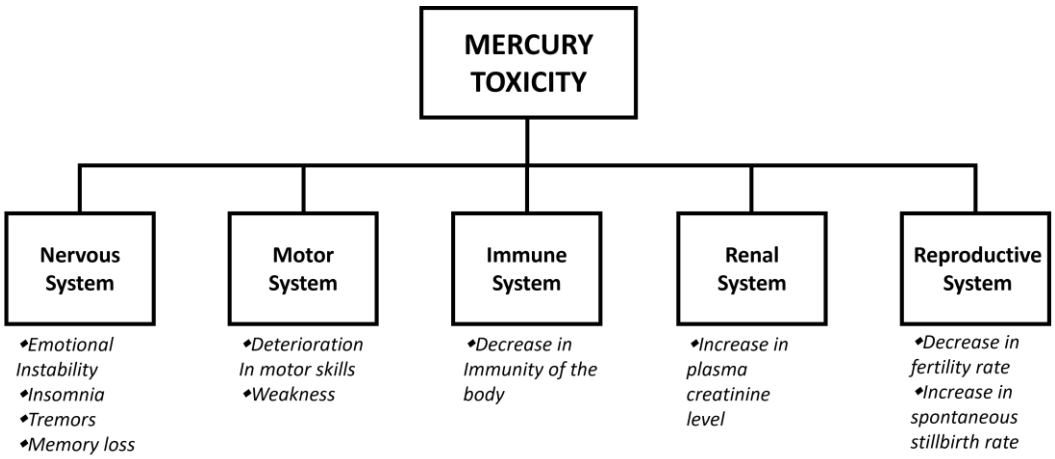


Figure 1-5: Summary of notable toxic effects of mercury on humans[72].

Methylmercury have been demonstrated to be able to inhibit DNA and RNA synthesis[73], inhibit protein synthesis, affect protein posttranslational modifications[74], affect cell adhesion and repulsion[75], and affect calcium homeostasis[75]. while there are attempts to study the mechanism of methylmercury toxicity, these studies have generally been limited to examination of cell culture systems and the underlying mechanism for toxicity remains largely unknown[75]. One of the established mechanisms for methylmercury toxicity stem from the ability of methylmercury to react with thiols such as cysteine and glutathione[75, 76]. The reaction with glutathione can cause depletion of glutathione and induce oxidative

stress, and the reaction with cysteine groups can lead to inhibition of critical proteins and in turn induce adverse effects.

Chromium is typically found in three states in the environment: elemental (Cr^0), trivalent (Cr^{III}) and hexavalent (Cr^{VI}). Cr^{VI} is most discussed as an environmental contaminant due to its carcinogenic properties, while Cr^{III} is considered non-toxic. The reason for the difference in toxicity between the two species is that the cell permeability of Cr^{III} is poor while Cr^{VI} can enter the cells and interact with DNA, forming Cr-DNA adducts and leading to chromosomal breaks and mutations (Figure 1-6) [77]. Studies have shown that exposure of animals to drinking water contaminated by Cr^{VI} induced digestive tract tumors due to its ability to cause DNA damage[78, 79].

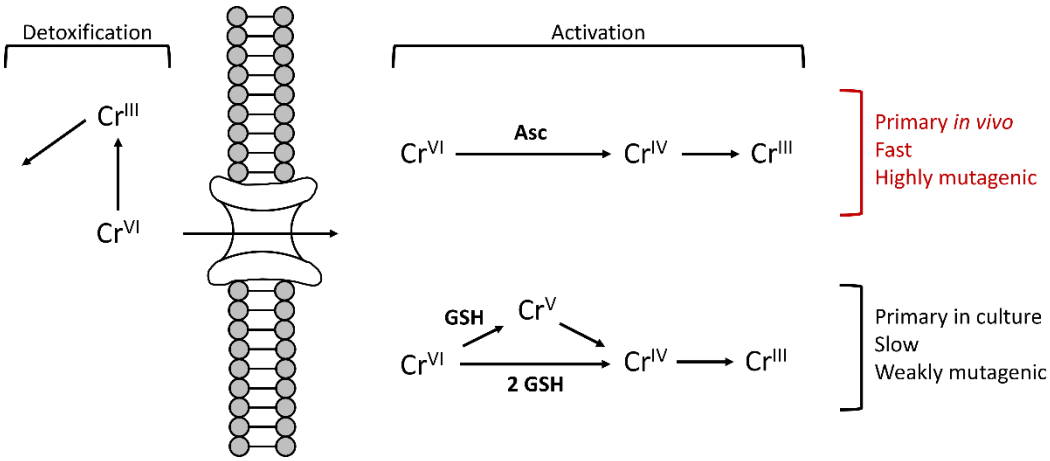


Figure 1-6: Illustration of the primary chromium carcinogenicity pathway[77]. Extracellular reduction detoxifies as poorly cell-permeable Cr^{III} is formed; cellular reduction however activates toxicity as Cr^{III} can form mutagenic Cr-DNA adducts.

Lastly, cadmium, a transition metal commonly found in its bivalent form, can be taken up by both aquatic and terrestrial plants and animals from water and soil. Cadmium has

no known biological functions in humans, and is regarded as carcinogenic[80]. Besides carcinogenicity, chronic exposure to cadmium was shown to lead to kidney[81] and bone[82] damage, neurobehavioral[83] and immune systems[84] impairment, and developmental defects[85]. Usually, cadmium does not present great food safety risks due to its low absorption from the gastrointestinal tracts, which is even further decreased in zinc and iron rich foods as they compete with cadmium for absorption[80]. However, excretion of cadmium from the body is very poor, and therefore can still pose great health hazards on the body if there is chronic exposure[86].

Overall, all of the mentioned contaminants pose great food safety risks, and evaluation of their possible accumulation in deep-frying oil is justifiably needed.

1.4. Objectives

This project focuses on addressing the possible accumulation of heavy metals, and the potential change in species of arsenic during deep-frying under conditions comparable to the practice of the local food trade, such as the deep-frying parameters and materials used. The experimental particulars are as follows:

1) To evaluate whether accumulation of heavy metals is possible by deep-frying seafood of high heavy metal concentrations, provisionally only oysters, in the same pan of oil repeatedly and checking whether the heavy metal content of the deep-frying oil increased over time using inductively coupled plasma-mass spectrometry (ICP-MS).

2) To investigate the accumulation mechanism by isolating the components involved in the deep-frying process and determining their heavy metal content.

3) To evaluate the applicability of the accumulation mechanism by using different food types and observing if the accumulation is seen in other types of food, and if the mechanism is similar.

4) To investigate the possible changes in arsenic species distribution in foodstuff due to deep-frying and in turn identify potential increase in risk of consumption by liquid chromatography (LC) coupled with ICP-MS.

Chapter 2:Accumulation of arsenic in deep-frying oil

2.1. Introduction

Possible accumulation of heavy metals in deep-frying oil is the main objective of this study. For reference, the total arsenic and lead limits in edible oils as established by the Hong Kong government in the Food Adulteration (Metallic Contamination) Regulations[87] are both 0.1 parts per million (ppm), equating to 100 parts per billion (ppb). To evaluate not only the health risks but also the possibility of the food trade violating the regulations unknowingly by repeatedly using the same pan of deep-frying oil, a set of experiments mimicking typical food trade practice was designed to see whether the oil used can exceed the legislation limit.

Deep-frying parameters in this study were selected based on recommendations on deep-frying. Many guidelines and suggestions were made available by governing bodies and institutions, with the most comprehensive one being the Optimum deep-frying Recommendations by the German Society for Fat Science[11] and the most recently published one being the Trade Guidelines on the Use of Deep-frying Oil by the Hong Kong Government in 2019[10]. These two documents included generic knowledge such as introduction of the deep-frying operations to specific recommendations such as deep-frying oil temperatures, types of edible oil suitable for deep-frying, food treatment etc.
The deep-fried food chosen for this study should be a type with high arsenic and lead concentration to promote the accumulation of these contaminants if any. Oyster was selected as the seafood to be deep-fried due to it being a common type of deep-fried food out of bivalves species while having high arsenic and lead content[88]. Possible effects of the type of edible oil used on the accumulation of heavy metals were also studied by

comparing the accumulation trends of soybean oil and rapeseed oil, two of the most consumed types of edible oil worldwide[89], under the same experimental parameters.

Samples acquired require total arsenic and lead analyses, involving the digestion of samples followed by elemental analysis. This study employs the pressure digestion technique for digestion and inductively coupled plasma-mass spectrometry (ICP-MS) for elemental analysis.

ICP-MS is a powerful analytical technique typically used for elemental analysis at trace concentrations. Although a number of older techniques such as inductively coupled plasma optical emission spectroscopy are still in use by some laboratories, there has been a shift toward ICP-MS due to its high sensitivity[90]. The basic instrumental components of an ICP-MS are shown in Figure 2-1. Ionization using ICP is a unique technique because it is element specific. Using arsenic as an example, when one uses ICP all arsenicals are atomized and is detected as As (detected at 75 mass-to-charge ratio (*m/z*) when coupled with MS) no matter what chemical form the arsenic is in.

An internal standard (common choices include selenium and indium) is used for more accurate analyses. The ratios of the intensities of the target analytes and the internal standard peak are used for quantification to minimize the effects of factors such as instrumental fluctuations. With little interference and well resolved peaks, spike recoveries, detection limits and linearities etc. are often excellent and quantification can be easily achieved.

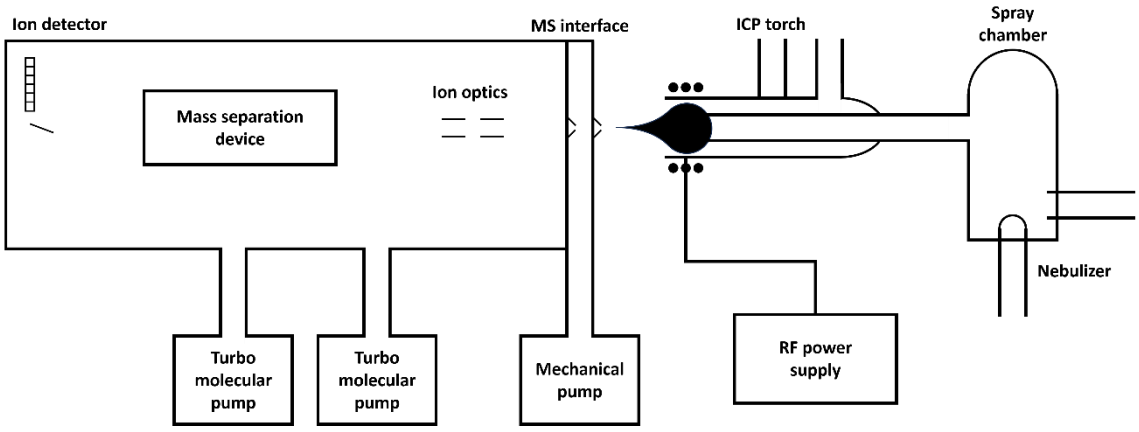


Figure 2-1: Basic instrumental components of an ICP-MS[91]. The sample is pumped into the nebulizer to give a fine aerosol. Fine droplets of the aerosol then enter the ICP torch to generate positively charged ions. The ions are then directed into the MS unit for measurement.

2.2. Experimental

2.2.1. Materials and chemicals

Unless otherwise specified, ultrapure water generated with Milli-Q® Direct Water Purification System produced by Merck (Burlington, Massachusetts) was used throughout the entire study.

Kitayoshi Suisan (北吉シーフーズ) frozen oysters (Figure 2-2) from Japan were purchased from local supermarkets and used in this experiment. The deep-frying oils used include rapeseed oil and soybean oil as they were oils commonly used for deep-frying operations by the food trade in Hong Kong. The rapeseed oil used was Hop Hing (合興) SS Rapeseed Oil (SS 菜籽油) (Figure 2-2) and the soybean oil was Sam Hei (三喜) Golden Happy Premium Cooking Oil (金喜上等食油) (Figure 2-2), both purchased from Ball Kee grocery store (波記糧食) in Tsim Sha Tsui, Hong Kong.

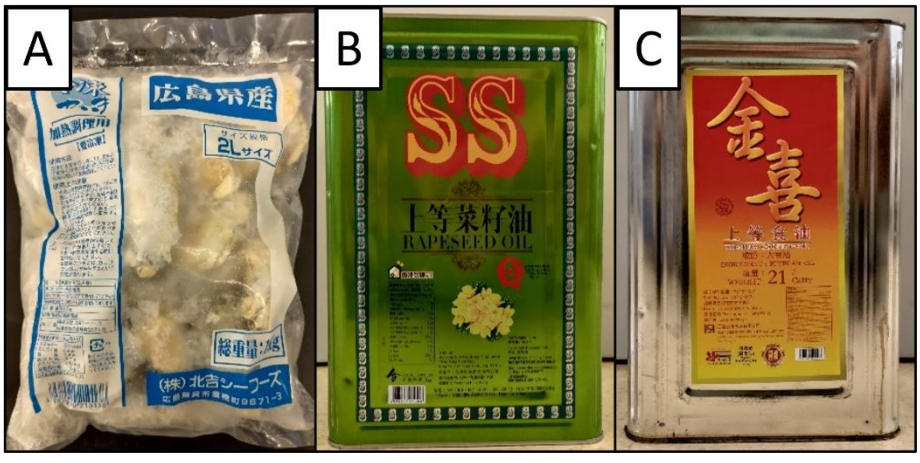


Figure 2-2: Photos of frozen Japanese oysters (A), rapeseed oil (B), and soybean oil (C) used in this study.

Arsenic standard for ICP, lead standard for ICP, and indium standard for ICP used were produced by Sigma-Aldrich (St. Louis, Texas). Trace metal grade nitric acid used was produced by Anaqua Chemicals Supply (Cleveland, Ohio). Hydrogen peroxide solution (for ultra traces analysis) used was produced by Fisher Chemicals (Leicestershire, United Kingdom).

2.2.2. Sample preparation

Stock solutions and foodstuffs were stored according to suppliers' instructions. Working standard mixes (arsenic and lead) and internal standard solution (indium) used for calibration were prepared by dilution of stock solutions.

2.2.3. Deep-frying operation

Preparation before deep-frying operations including defrosting oysters and cleaning utensils beforehand to minimize introduction of moisture into the deep-frying oil, mimicking typical food trade practice. Frozen oysters were defrosted by placing in a 4 °C refrigerator until defrosting is completed. The oysters were placed on a strainer to drain liquid coming from the defrosting process. All utensils including strainer, cooking tongs, ladle and oil pan were cleaned in advance to ensure each of the items had ample time to be thoroughly air-dried.

Deep-frying parameters were selected based on suggestions and recommendations published by institutions and governing bodies[10, 11]. IKA C-MAG HS7 hot plate was used

for heating up the deep-frying oil, and the accessory ETS-D5 electronic contact thermometer was used for deep-frying oil temperature control (Figure 2-3A). The deep-frying temperature was set at 170 °C. The recommended food-to-oil ratio was 1:20, and thus 400 g of cooking oil was used for deep-frying oysters of weights of roughly 20 g. Deep-frying cycles were determined to be 3 minutes during test runs, in which all deep-fried oysters were fully cooked.

Deep-frying operations were completed in fume hood to ensure sufficient ventilation. Deep-frying oil was heated to 170 °C. For each cycle, one oyster was placed into the deep-frying oil with care and was allowed to deep-fry for 3 minutes. After deep-frying is completed, the deep-fried oyster was removed from the deep-frying oil using a strainer and allowed to rest for 1 minute to strain any excess oil from the oyster. The oyster was weighed both before and after deep-frying to measure the mass loss caused by the deep-frying process. If sampling is needed for the cycle, a ladle is used for mixing and sampling the deep-frying oil. The oil is allowed to be heated up to 170 °C again before subsequent deep-frying cycles, if any. The test was also repeated with the practice of straining the oil after each cycle (before sampling) to investigate the effects of straining on arsenic accumulation. The fine strainer (mesh number ~50; mesh size ~0.297 mm) used is shown in Figure 2-3B.

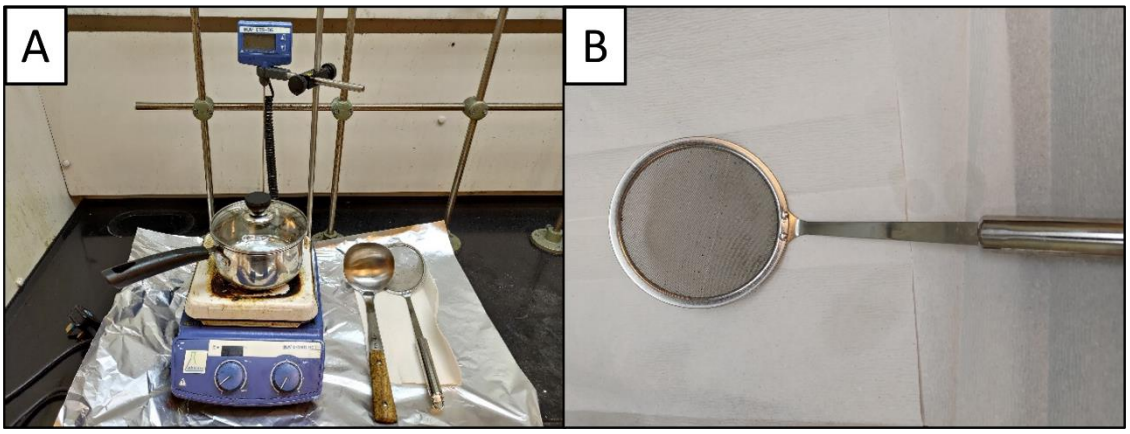


Figure 2-3: Deep-frying setup (A) and the fine strainer (B) used for straining oil.

Sampling was designed to be intermittent to minimize oil loss due to sampling. Roughly 5 mL of sample is taken after 0, 1, 2, 4, 8, 16, 24, 32, 48, 64 cycles. Sampling was designed to be more frequent towards the beginning to monitor potential rapid changes in oil quality in the early stages of deep-frying. Rate of oil deterioration and potential accumulation of contaminants should have stabilized during later deep-frying cycles, and thus sampling was designed to be less frequent. The residues filtered from the oil were also collected for analysis at cycles 8, 16, 24, 32, 48, and 64. Residues were not sampled at cycles 0, 1, 2, and 4 because the formation of residue was insignificant during earlier cycles and the amounts of residues yielded were insufficient for analysis.

A Testo 270 handheld TPC device was used to record the TPC content of the deep-frying oil at the end of each set of experiment (i.e. after 64 deep-frying cycles). The handheld TPC device has resolution of 0.5% and a maximum detection limit of 40%. This is to provide an estimate of the degree of oil deterioration for comparison between sets of experiments.

2.2.4. ICP-MS analysis

Samples were initially studied using semiquantitative analysis, and subsequently studied using fully quantitative analysis for arsenic based on the findings. The samples were digested by microwave pressure digestion using Milestone Ethos Easy Microwave Digestion Platform with the Standard Kit-10 vessels. The pressure digestion method was developed with reference to standard method European Standard EN 1380529 and the ICP-MS analysis method was developed with reference to European Standard EN 1576330. 2 mL of concentrated nitric acid (HNO3) and 1 mL of 30% hydrogen dioxide (H2O2) were added to 0.2 g for oil or residue sample, and 1 g for food sample, for the digestion. To prevent overpressure during the microwave digestion, samples were allowed to pre-digest for 10 minutes to allow the evolved gases to escape before starting the digestion procedure. The digestion program was set to gradually increase the temperature to 180 °C and holding for 25 minutes. After the digestion, the collected digest including the rinse was marked up to 20 mL. 0.1 mL of 0.1 mg/kg indium standard solution was added to each sample as the internal standard. The calibration curves were also prepared using the same volumes of solutions with matrix match, where for each individual working standard solution, 2 mL of the trace metal grade concentrated HNO3 was added and marked up to 20 mL, and 0.1 mL of 0.1 mg/kg indium standard solution was added. The samples were analysed using the Agilent 7900 ICP-MS system in helium mode, where helium was used as the reaction/collision gas. The ICP-MS system was tuned using the Tuning Solution for ICP-MS produced by Agilent before each run. The conditions of the ICPMS were as follows: RF power: 1550W; nebulizer gas (argon) flow rate: 0.9 L/min; auxiliary gas (argon) flow: 0.9 L/min; plasma (argon) gas flow: 15 L/min; reaction gas flow (helium): 4 mL/min; dwell time: 100 ms; nebulizer: glass concentric

type. Each sample digested and tested in triplicates. Semiquantitative analysis of elements was performed using the quick scan feature IntelliQuant from Agilent, where the full mass spectrum was acquired from *m/z* 2 to 260 in 2 seconds and the collected data for all isotopes of elements allowed for semiquantitative measurements of up to 78 elements. For fully quantitative analysis of arsenic, the data were acquired at *m/z* 75, and the calibration curve was constructed using nine arsenic standard solutions with concentrations of 0, 0.05, 0.1, 0.2, 0.5, 1, 1.5, 2.5, and 5 μg/kg, with indium (*m/z* 115) used as the internal standard. Samples with arsenic concentrations beyond the calibration range were diluted to give concentrations within the calibration range for measurements with good accuracies. Details on the operating conditions of the ICP-MS can be found in Table S-3.

2.3. Results and discussion

2.3.1. General Observations

Repeated use of the deep-frying oil caused noticeable quality changes in the deep-frying oil, reaching a TPC value of >40% at the end of the experiments after 64 deep-frying cycles. The colour of the deep-frying oil gradually changed from light yellow to brown to very dark brown, and the colour of the oysters deep-fried also demonstrated a similar change (Figure 2-4). Blackened residues started to accumulate in the oil, some sticking to the side and the bottom of the pan while finer pieces were suspended in the deep-frying oil. These residues will be discussed in more detail later.

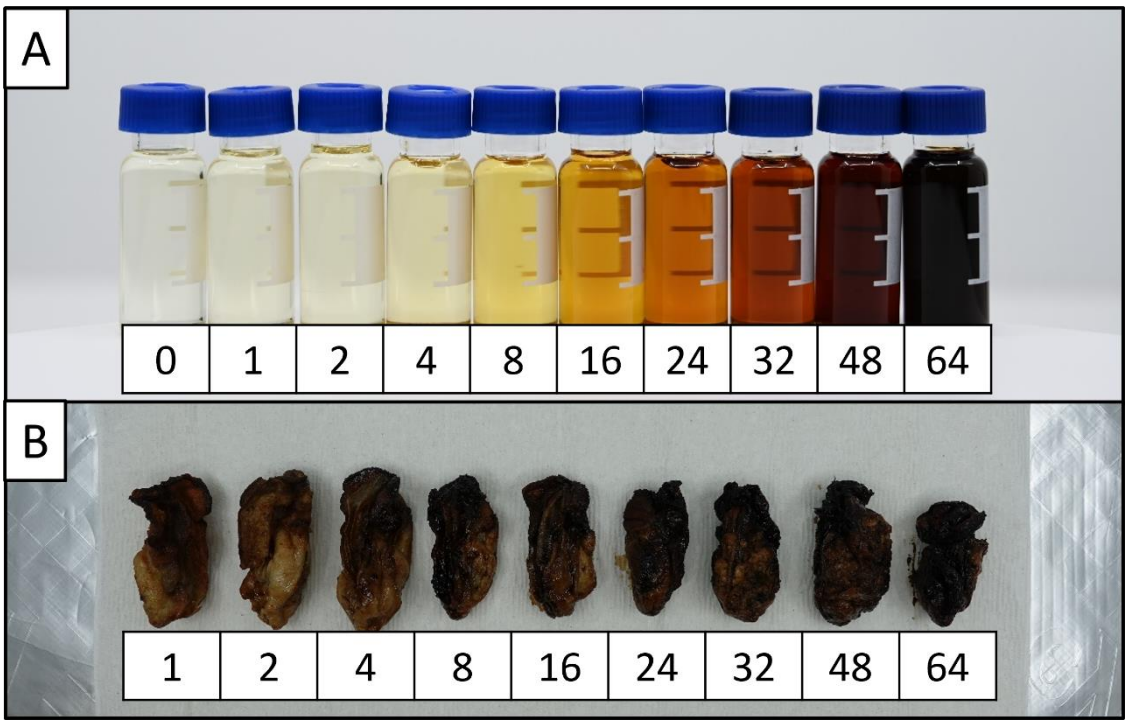


Figure 2-4: Colour change of deep-frying oil (A) and deep-fried food (B) over 64 deep-frying cycles. The cycle numbers of the samples from left to right are 0, 1, 2, 4, 8, 16, 24, 32, 48, and 64, while the oysters did not have a cycle number 0 sample.

2.3.2. Accumulation of arsenic in deep-frying oil

Deep-frying oysters in rapeseed oil without straining was investigated to see if heavy metals can accumulate in the deep-frying oil over time. Heavy metals that were below the limit of detection in the raw oysters were still undetectable, but arsenic concentrations increased rapidly with the number of deep-frying cycles. Notably, in addition to arsenic accumulation, accumulation of some elements found to be of higher levels in raw oysters. Using the IntelliQuant feature, it was found that potassium, sodium, magnesium, zinc, manganese, copper, strontium and silver were also at increased levels in the deep-frying oil (Figure 2-5), indicating that various elements in the deep-fried food could be accumulated in the deep-frying oil.

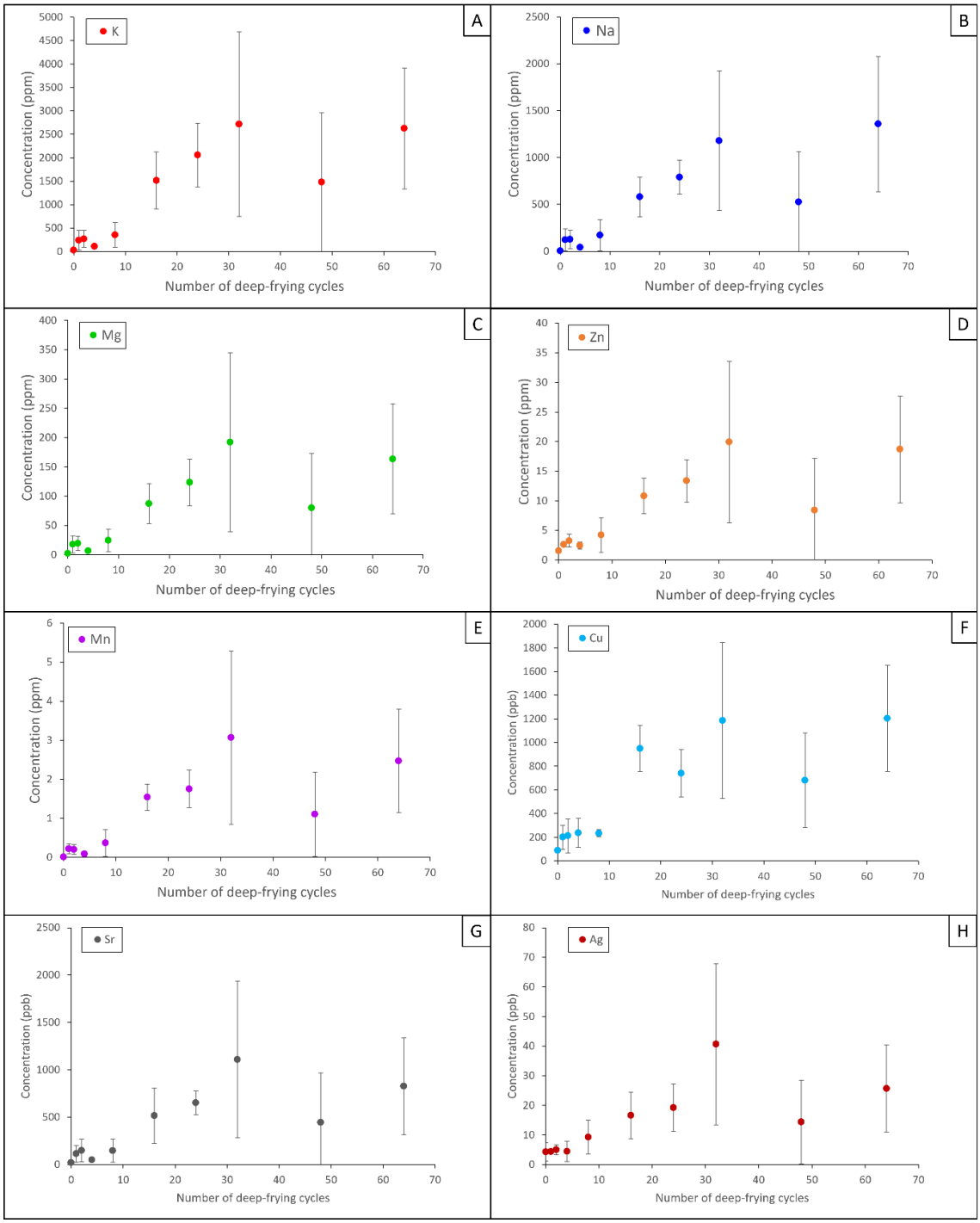


Figure 2-5: Concentrations of various elements in unstrained deep-frying oil versus number of deep-frying cycles.

Fully quantitative analysis was hence done for arsenic, and revealed a clear accumulation trend was observed as the concentrations increased rapidly with the number of deep-frying cycles. The concentrations of arsenic in the deep-frying oil accumulated to over

100 µg/kg, the regulation limit in Hong Kong[87] and the maximum level recommended by the Codex Alimentarius[92], within 16 cycles, and reached 567±133 µg/kg, which was over 5 times the limit, towards the end of the experiment.. It is therefore possible for the food trade to generate deep-frying oil that are illegal to use during deep-frying practices, in turn violating the regulations unknowingly.

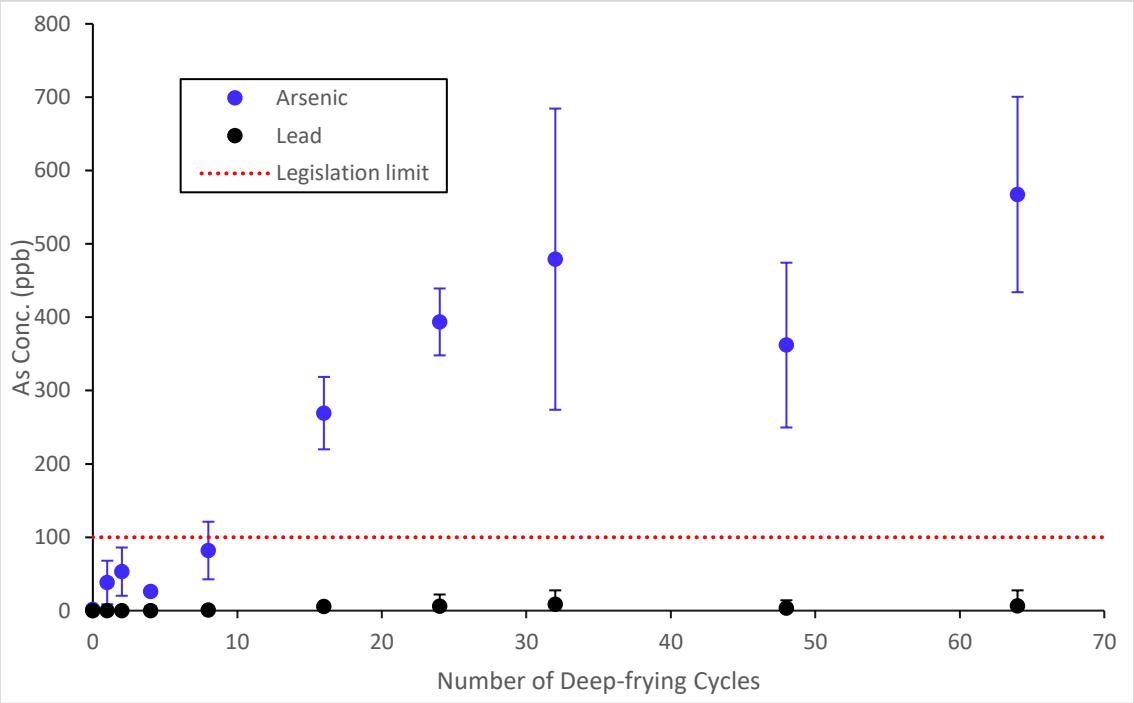


Figure 2-6: Arsenic and lead concentrations of unstrained deep-frying oil versus number of deep-frying cycles. The dotted line shows the legislation limit.

One notable observation was that the standard deviation between replicates were remarkably larger for the unstrained oil samples. Large standard deviation between replicates is most likely caused by inhomogeneity of samples, and the inhomogeneity was likely caused by the suspended residues that were unstrained. Thus, the study was repeated with straining by using a strainer to remove residues from the oil after each cycle

and before the sampling of oil. Other than the difference between straining and not straining, the two pans of oil were treated impartially.

Results from the strained deep-frying experiments are shown in Figure 2-6. Similar to the unstrained experiments, the lead concentrations were well below the legislation limit and at insignificant levels. However, the arsenic concentrations were much lower compared to the unstrained series (Figure 2-7) as the arsenic concentration only reached 277 ppb after 64 deep-frying cycles, roughly equalling a 50% reduction in arsenic concentration in the deep-frying oil. However, despite the 50% reduction in arsenic concentration, it was demonstrated that it is possible to reach the legislation limit of 100 ppb in edible oil even with straining. It was also noted that the standard deviations between sample replicates were significantly smaller in the strained series compared to the unstrained series.

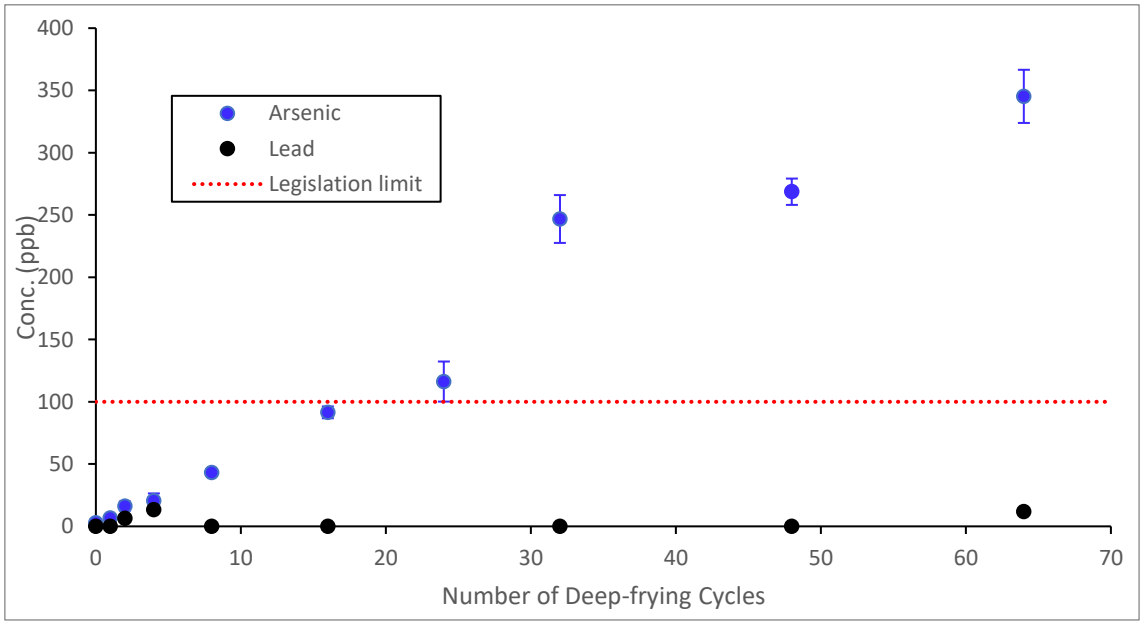


Figure 2-7: Arsenic and lead concentrations versus number of deep-frying cycles for the strained oil experiment.

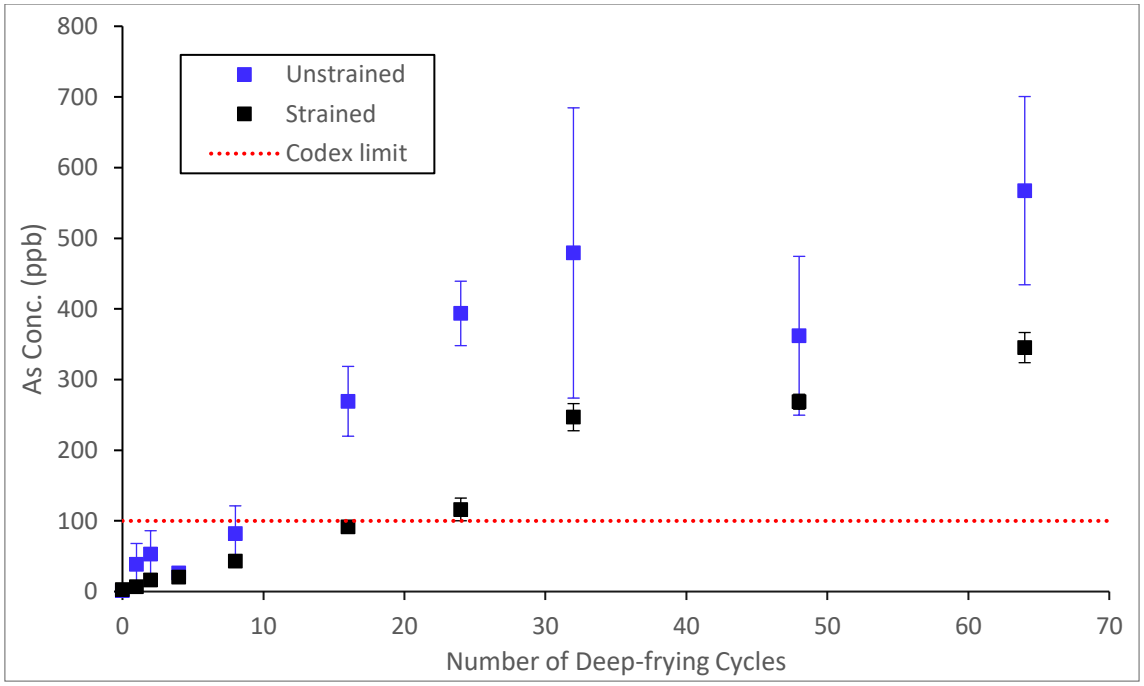


Figure 2-8: Comparison of arsenic concentrations of strained oil versus unstrained oil. Note the standard deviations between sample replicates were significantly smaller in the strained series compared to the unstrained series.

A comparative study was designed to see the effects of the type of oil on heavy metal accumulation rates. Soybean oil and rapeseed oil, two of the most consumed types of edible oil worldwide[89], were used to repeatedly deep-fry oysters for 64 cycles using the same parameters. The soybean oil and rapeseed oil data showed minimal differences in terms of deterioration rate, both colour and TPC value. Arsenic accumulation rate were slightly higher for rapeseed oil compared to soybean oil, but still within a similar range (Figure 2-9). Because the oil quality changes were similar, replicate experiments using multiple types of oil was deemed unnecessary and all subsequent deep-frying experiments were done using one type of oil only. Rapeseed oil is chosen instead of soybean oil beacuse rapeseed oil is recommended over soybean oil for deep-frying purposes by the Trade Guidelines on the Use of Deep-frying Oil published by the Centre for Food Safety

(CFS) of the HKSAR Government[10], and thus using rapeseed oil should be more representative of the trade practice in Hong Kong.

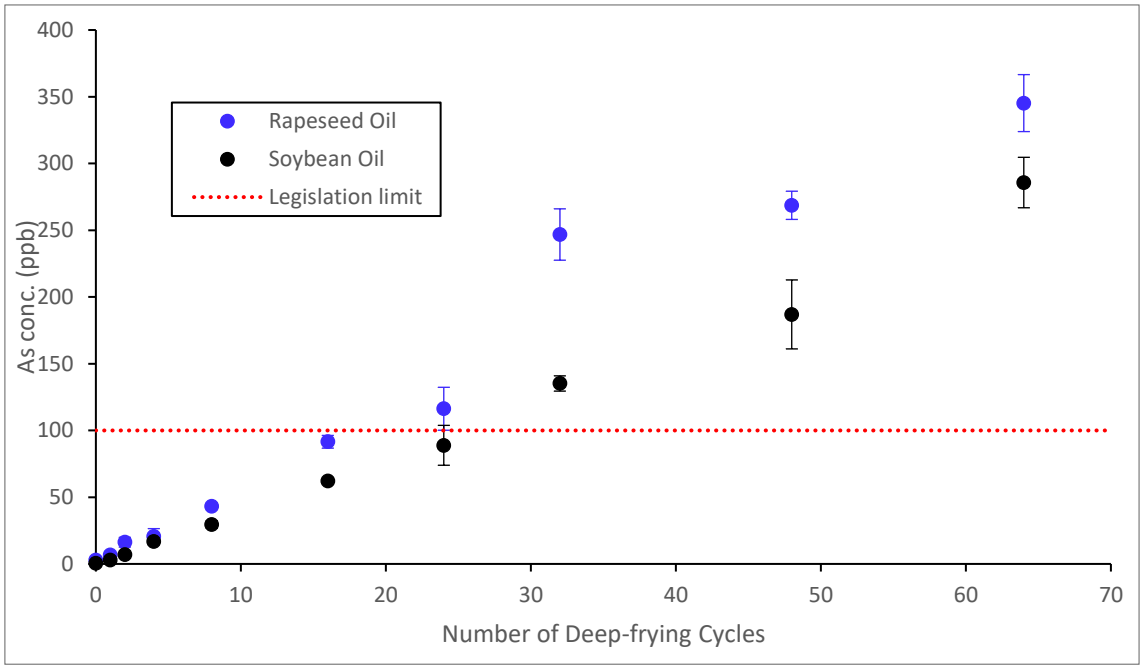


Figure 2-9: Comparison of arsenic levels in rapeseed oil and soybean oil under the same deep-frying conditions.

2.3.3. Elemental analysis of deep-frying residues

Since removing the residues considerably reduced the arsenic concentration in oil as well as the fluctuations between sample replicates, it is likely that the residues contained high levels of arsenic that can influence the detected concentrations. To verify the hypothesis, residues from the strained oil were collected and examined in detail. These residues appeared to be carbonaceous and tended to form clusters of a few millimetres in diameter (Figure 2-8A). It was observed that very little carbonaceous residue was formed when the oil is fresh; the amount of carbonaceous residue formed was not sufficient for analysis

for cycles 0, 1, 2, and 4. However, the amount of carbonaceous residues formed were substantially more during later cycles compared to earlier cycles (Figure 2-8B). Total arsenic contents of these residues were analysed, and it was found that the arsenic concentrations of these residues were magnitudes higher than that of the deep-frying oil (Figure 2-9). The average concentration of these residues was at approximately 6.7 ppm, which is even higher than that of tested fresh oysters at roughly 1.8 ppm; a similar phenomenon was observed with lead where the concentrations are higher in residues (0.39 ppm) compared to in fresh oysters (0.13 ppm). The high arsenic and lead content suggested that the food safety risk associated with these residues may be more significant than that with the deep-frying oil. More importantly, the enrichment of lead in residues indicated that enrichment effect is not limited to arsenic. It is likely that only arsenic accumulation is observed because of the high arsenic concentration in oysters, and accumulation patterns would be observed for other heavy metals if the concentrations in the deep-fried food were higher. Semiquantitative results (Table 2-1) were found to be consistent with the speculation, as multiple elements were found to be at higher concentrations in residues compared to in the raw oysters, including all of the five heavy metals that commonly induced human poisoning. Notably, the concentration of mercury was found to be higher in residues but the standard deviation between triplicate measurements was large. This is possibly due to the volatility of mercury at elevated temperatures[22, 93], leading to easy evaporation and in turn fluctuation in sample replicates. The semiquantitative results, both for the residues and the deep-frying oil from different cycles, were consistent with the study by Artz et al.[20] where iron was found to accumulate in oil, and the study by Atia et al.[22] where heavy metal in food was believed to be lost to the cooking oil. Transfer of heavy metal as well as other elements is demonstrated to be possible in this study.

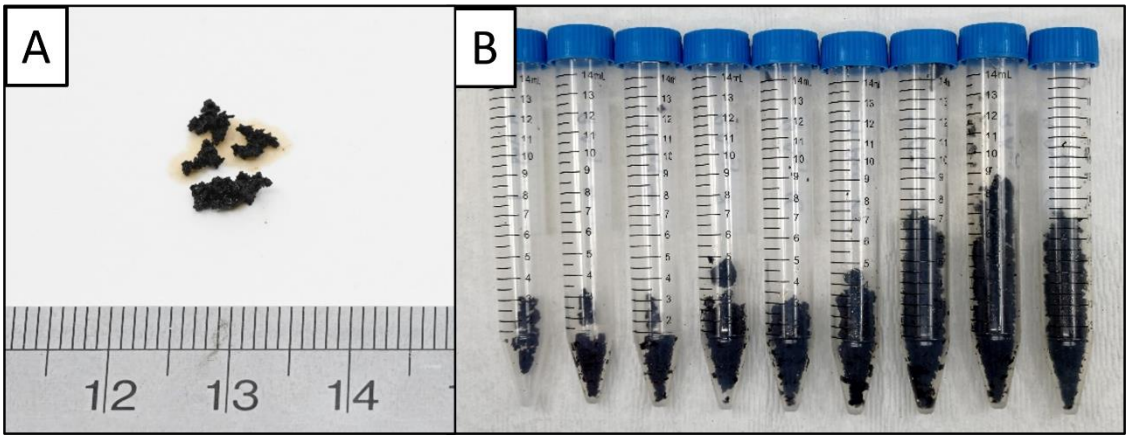


Figure 2-10: (A)**:** Size and appearance of the strained carbonaceous residues and (B): comparison of amount of residues yielded from cycle 1, 2, 4, 8, 16, 24, 32, 48, 64 from left to right. The amount of residues yielded from later cycles is notably greater than that yielded from earlier cycles.

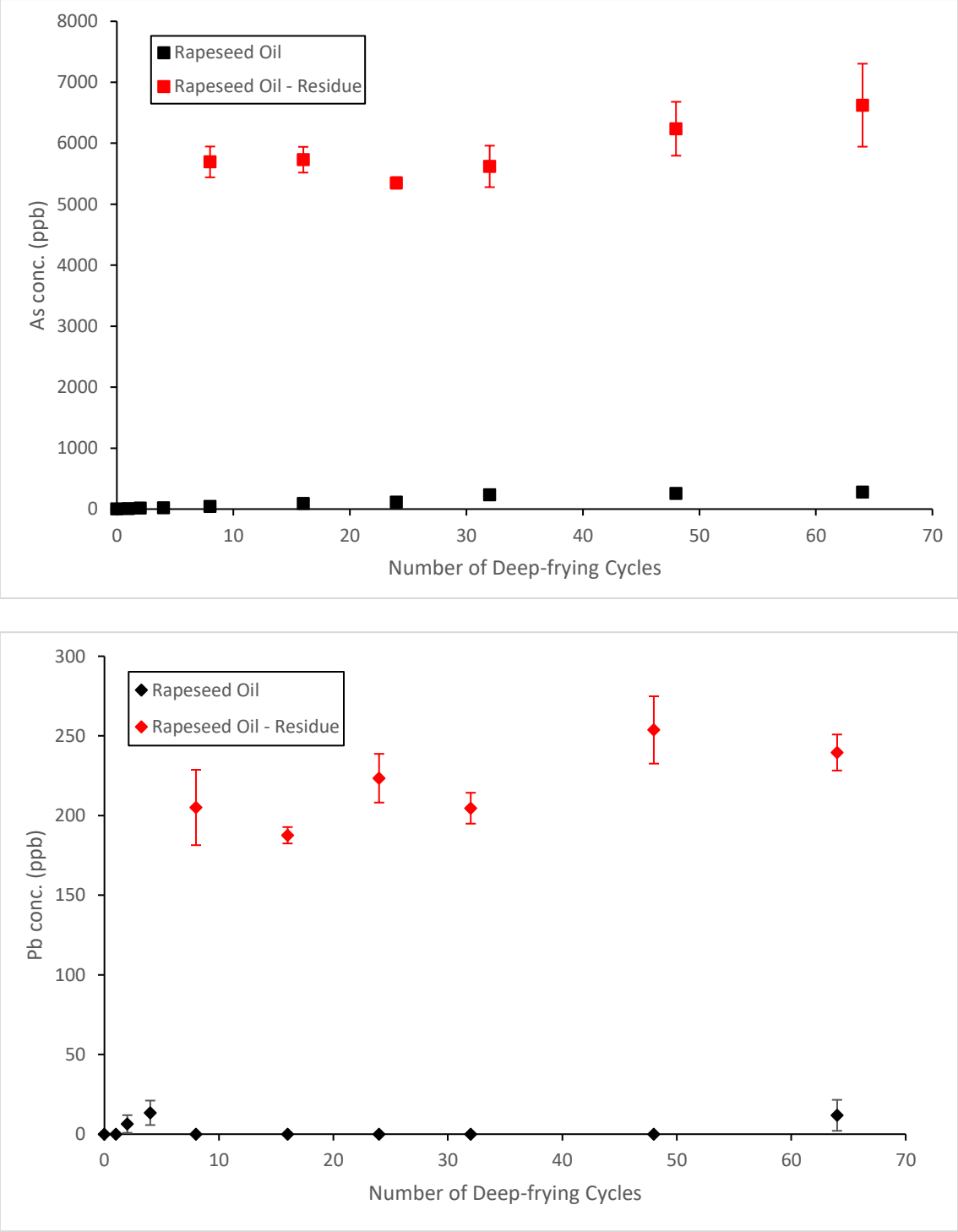


Figure 2-11: Plots of arsenic concentration (top) and lead concentration (bottom) versus number of deep-frying cycles. These show the contrast between the concentrations in oil versus in the carbonaceous residues.

Table 2-1: Concentrations of elements commonly found in oysters and five heavy metals in raw oysters and deep-frying residues, obtained by semiquantitative analysis.

	Raw oyster	**Oyster deep-frying residues**
Na (g/kg)	3.0±0.3	42.0±13.4
Mg (g/kg)	0.8±0.0	7.2±2.2
Cl (g/kg)	4.9±0.3	70.7±23.6
K (g/kg)	6.8±0.3	102±52.5
Cr (µg/kg)	157±89.1	528±220
Mn (mg/kg)	19.3±0.3	102±47.5
Cu (mg/kg)	12.3±0.5	45.3±10.7
Zn (mg/kg)	0.30±0.02	0.81±0.22
As (mg/kg)	5.1±0.3	43.2±8.4
Sr (mg/kg)	5.2±0.4	43.2±13.1
Ag (µg/kg)	372±22.1	1236±53.7
Cd (µg/kg)	592±59.7	2182±148
Hg (µg/kg)	5.5±1.5	177±199
Pb (µg/kg)	376±12.7	1470±367

Accumulation of arsenic in strained oil samples may also be due to residues in oil. After allowing samples from the strained oil experiment to settle, a thin precipitated layer of fine particles was deposited at the bottom of the container (Figure 2-10). The precipitation required prolonged periods of time, and the exact time needed may depend on various factors such as particle size, viscosity of oil, and storage condition. Given that the arsenic and lead concentrations of the residues are magnitudes higher than that of the deep-frying oil, it is possible that the fine suspended residues were the main source of arsenic and lead content in the deep-frying oil.

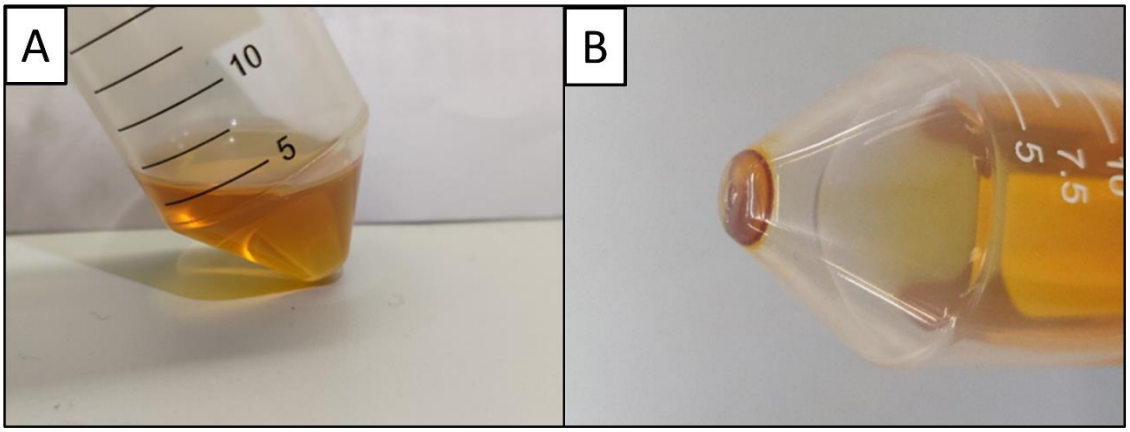


Figure 2-12: Deep-frying oil sample immediately after collection (A) and after allowing to rest for a prolonged period of time (B). Sample appears to be clear after straining as shown in A, but precipitated fine particles were observed later as shown in B.

2.4. Conclusions

Accumulation of arsenic was evident in the oil, especially for the unstrained oil experiment set; accumulation of lead on the other hand was not significant. Unstrained oil sample replicates demonstrated exaggerated fluctuations and was found to be likely due to the inhomogeneity of the sample, as the arsenic and lead concentrations of the strained residues were profoundly higher than that of the deep-frying oil. The residues and the accumulation mechanisms needed to be investigated to fully evaluate possible food risks.

Chapter 3: Investigation of accumulation mechanism in deep-frying oil

3.1. Introduction

Accumulation of arsenic appeared to be most significant within the residues formed; if the residues were not strained from the oil the total amount of arsenic in the oil pan can build rapidly. Investigation of the accumulation mechanism thus demand the study of these residues and how the arsenic is deposited onto them preferentially. The carbonaceous residues evidently originated from the oysters since no residues were formed if the oil was heated without the addition of oysters. Thus, it is most likely that the residues are formed by leaching of mass from oyster into the oil during deep-frying. Liquid seeped from oysters can contain molecules such as proteins that can denature and brown under high heat, giving dark brown residues as the droplets are dehydrated and crusts are formed.

Evidence in the literature suggests that liquid originating from oysters can contain mass leached from the oyster. Shucking liquid, or oyster liquor, is the liquid collected when oysters are shucked from their shells. Researchers took interest in the shucking liquid since it is often used in the preservation of oysters by submerging shucked oysters in the liquid to preserve its natural flavour[94]. Shucking liquid contains proteins, nonprotein nitrogenous components and other solids[94] as well as heavy metals[95]. Even if the shucking liquid is washed off the oyster, wash water from after the oysters had been shucked and washed would still contain heavy metals albeit at a lower concentration[95], showing that liquid leached from oysters can contain heavy metals. Thus, it is possible that the liquid leached from oysters is a source of the heavy metals seen in the deep-frying experiments of this study.

Formation of more residues as the oil deteriorates can be explained by the surfactant theory of frying first proposed by Blumenthal[5]. He hypothesized that surfactant materials are formed in the oil as a result of degradation, and the surfactant increases the contact between the oil and the food. This causes higher dehydration in the surface, and water migration from the core to the surface of the food. Despite recent studies refuting the hypothesis[96] and suggesting the higher dehydration is due to the increased viscosity of deteriorated oil, the fact remains that dehydration and oil uptake is increased as the oil deteriorates.

Another possible mechanism for the accumulation of heavy metals in the residues is that the heavy metals are first leached into the oil. However, they are adsorbed by the residues, therefore causing a concentration effect in the residues and the "clean up" of the oil, much like how chemical adsorbents can remove certain materials from a solution. Metallic ions are theoretically more hydrophilic compared to the oil. If there are hydrophilic interactions between the surface of the residues and the heavy metals, it can lead to a strong affinity towards the residues compared to the oil and therefore the ions in the oil can be "picked up" by the residues. Regardless of the nature of the interaction between the residue and the heavy metals, adsorption and in turn concentration of contaminants onto residues is plausible.

Herein two mechanisms leading to the high concentrations of heavy metals are proposed. The first proposed mechanism (Figure 3-1A) is that the carbonaceous residues formed are from the liquid droplets coming from the oyster. As the droplets come into contact with the oil, moisture is rapidly removed due to the high temperatures, and a crust is formed

on the outer surface of microdroplets. This encapsulates heavy metals that was within the liquid from oysters, and thus producing residues of high heavy metal concentration.

The second proposed mechanism (Figure 3-1B) is that the heavy metals are first leached into the oil, and they have a strong affinity towards the residues formed during deep-frying. This affinity would result in the adsorption of heavy metals onto or into the residues, thus giving residues of high heavy metal concentration.

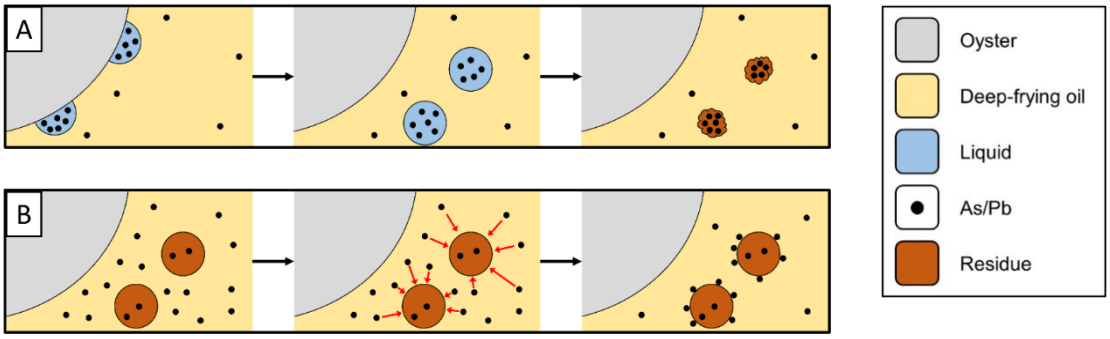


Figure 3-1: The (A) first proposed mechanism and the (B) second proposed mechanism of the formation of residues with high heavy metal levels. The figure key applies for both figures.

By better understanding the mechanism behind the accumulation of heavy metals, we can hopefully devise means to attenuate the accumulation rates and hence reduce the associated risk of consumption.

3.2. Experimental

3.2.1. Materials and chemicals

Frozen oysters and rapeseed oil used were the same as described in Chapter 2. Tempura flour used for arsenic adsorption study and the battered food study was Welna Tempura Batter Mix produced by Nisshin Seifun Group (Gunma, Japan). Conostan Oil Analysis Standards Custom Blend (Al, As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Zn) used for arsenic adsorption study was produced by SCP Science (Quebec, Canada).

3.2.2. SEM analysis of deep-frying residue

Carbonaceous residues collected were analysed using the JEOL Model JSM-6490 scanning electron microscopy (SEM) system. The residues were imaged to study the surface morphology. Energy dispersive X-ray spectroscopy (EDX)) using the same SEM system was also employed for elemental analysis of the residues. Carbonaceous residues were also analysed for their total arsenic and lead content using the same method as described in Chapter 2.2.4.

3.2.3. Analysis of defrost liquid

Frozen oysters were defrosted by placing in a 4 °C refrigerator until defrosting is completed. The oysters were placed on a strainer to drain liquid coming from the defrosting process. The liquid collected, named defrost liquid, was first analysed for its moisture, arsenic and lead content. The liquid was freeze-dried using the Martin Christ freeze-dryer Alpha 1-4 LD plus to give solids and analysed for arsenic and lead content.

The defrost liquid was also deep-fried to see if the morphology of the carbonaceous liquid generated would match the carbonaceous residues collected in the experiments in Chapter 2. The oil was first heated to 170 °C. 5 mL of the defrost liquid was then added to the oil and allowed to deep-fry until all the water has been evaporated. The resulting residues were then strained from the oil and later analysed.

3.2.4. Arsenic adsorption study

The arsenic adsorption study was done in two parts to test whether the arsenic in oil would be absorbed or adsorbed by residues introduced into the oil.

In the first part, fresh deep-frying oil was spiked with Conostan standard to give a high initial concentration of dissolved arsenic in oil. Then the oil was heated to 170 °C and 5 mL of the tempura batter mix was added to the oil and allowed to deep-fry for 3 minutes. The yielded batter pieces and the oil were sampled for analysis. A total of 8 deep-frying cycles were completed for this study.

In the second part, abused deep-frying oil was used to deep-fry batter mix. Oil obtained after repeatedly deep-frying oysters for 64 cycles was heated to 170 °C and 5 mL of the tempura batter mix was added to the oil and allowed to deep-fry for 3 minutes. The yielded batter pieces and the oil were sampled for analysis.

3.2.5. Battered food study

To use flour or batter to coat the food being deep-fried is a common practice in the food trade to improve texture. A study using the same deep-frying and sampling parameters but with battered food was completed to investigate the effects of the use of batter on the accumulation of heavy metals.

The batter used was a pre-made tempura batter mix to ensure the prepared batter is similar in composition to existing products on the market by following a standardized protocol. The use of pre-made batter mix also ensured minimal batch-to-batch variability as only

water and the batter mix is required to prepare the batter, minimizing possible variations in natural ingredients such as eggs and milk. Batter is applied following the manufacturer's instructions of petting the food, in this case oysters, dry and then dipping it into the batter immediately before deep-frying while ensuring the entire surface is covered with batter.

3.3. Results and discussion

3.3.1. SEM analysis of deep-frying residue

Elemental composition and morphology were investigated using SEM and EDX for information regarding the formation of residues. Note that elemental composition determination by EDX is limited especially for elements of lower atomic number and its sensitivity is not very high (detection limit is around 10 ppm) [97] so it should only be used as a reference.

Elemental composition analysis results are summarized in Table 3-1, and an example spectrum is shown in Figure 3-2. Quantification is not reliable for elements of lower atomic numbers[98], but it is evident that the residues are mainly composed of carbon, nitrogen and oxygen with common ions such as sodium, potassium and chloride. SEM images revealed the surface morphology of the residues (Figure 3-3).

Table 3-1: Elemental composition analysis results by EDX.

Element	Weight%	Atomic%
C	65.3±2.2	73.0±2.0
O	29.4±2.2	24.7±2.0
Na	0.85±0.04	0.50±0.03
Mg	0.20±0.01	0.11±0.00
P	0.58±0.05	0.25±0.03
S	1.29±0.11	0.54±0.05
Cl	1.25±0.03	0.47±0.01
K	1.09±0.07	0.38±0.02

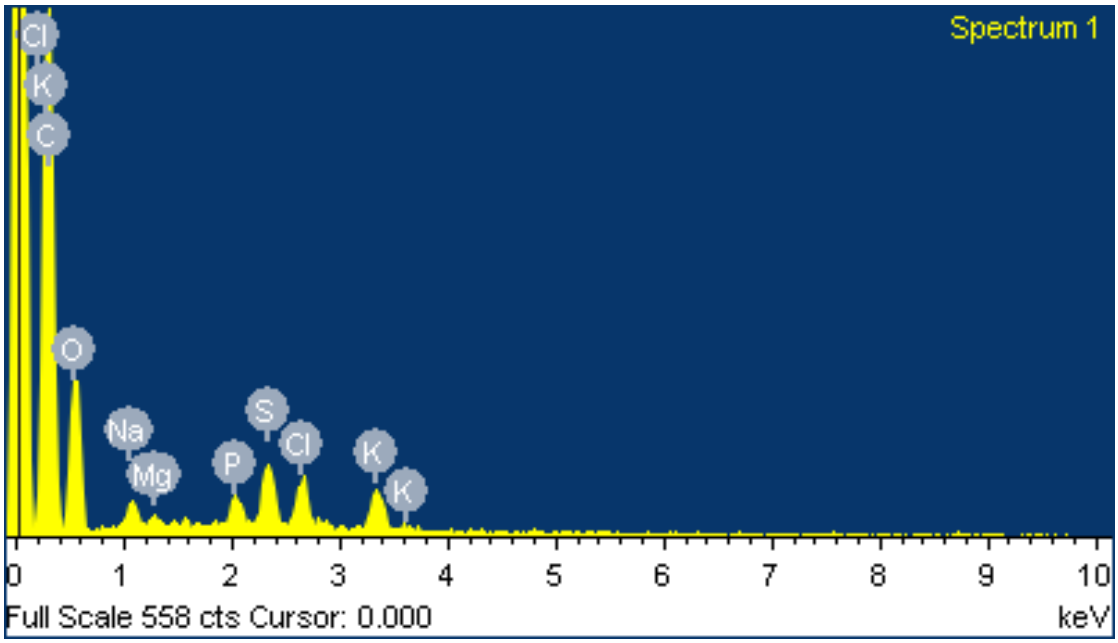


Figure 3-2: Example EDX elemental composition analysis spectrum.

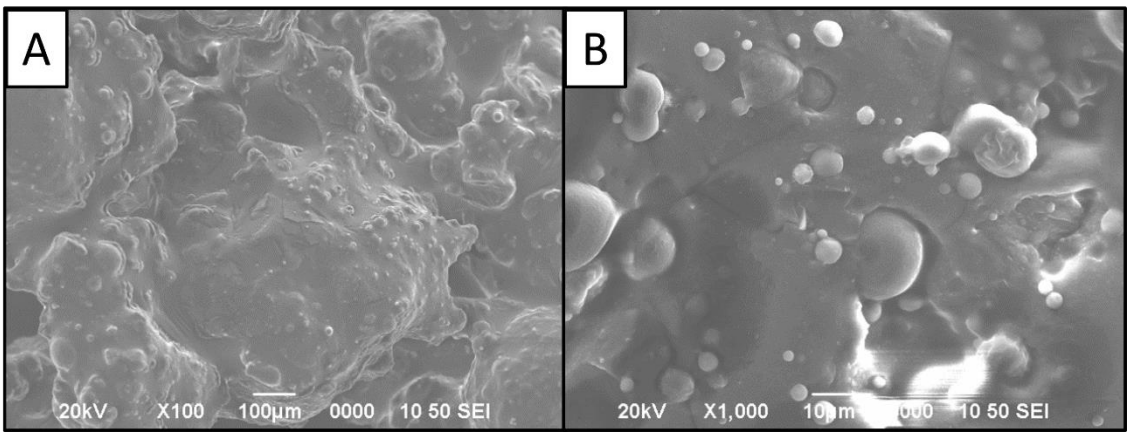


Figure 3-3: Surface morphology of carbonaceous residues at 100X (A) and 1000X (B) magnifications.

3.3.2. Analysis of defrost liquid

Defrost liquid was isolated from the oysters were tested for its arsenic and lead content. It was found that water consists roughly 90% of the defrost liquid, and to compare the residues would be dehydrated during the deep-frying process. Therefore, it is expected that the concentrations of arsenic and lead would be lower in the defrost liquid compared to the residues, and thus the defrost liquid was freeze-dried for a better comparison between the residues and the defrost liquid (Table 3-2). The freeze-dried defrost liquid had higher concentrations of arsenic and lead, but the two were in a similar range. The slight difference can be due to a number of contributing factors, including variation in the raw oysters tested, and the fact that the composition of liquid leached from oyster during deep-frying may only be similar and not identical to the defrosting liquid. Nevertheless, it was shown that liquid originating from the oysters can contain high levels of arsenic and lead, proving that the hypothesis “carbonaceous residues formed are from the liquid coming from the oyster” is a possible explanation. The residues having higher arsenic

concentrations is likely due to the enrichment caused by the evaporation of moisture during deep-frying.

Table 3-2: Arsenic and lead concentrations of defrost liquid, freeze-dried defrost liquid, carbonaceous residues and deep-fried defrost liquid. The "carbonaceous residues" are from the deep-frying experiments in Chapter 2, and "deep-fried defrost liquid" refers to residues yielded from deep-frying the defrost liquid.

	Arsenic concentration (ppm)	Lead concentration (ppm)
Defrost liquid	1.51±0.05	0.0418±0.0006
Freeze-dried defrost liquid	8.64±0.21	0.351±0.013
Carbonaceous residues	5.88±0.34	0.219±0.014
Deep-fried defrost liquid	10.3±0.2	0.309±0.046

Defrost liquid was also deep-fried to check whether the formed residues from the previous deep-frying experiments would resemble the residues from defrost liquid. Their appearances were very similar (Figure 3-4), and the concentration of the yielded residues were in a similar range to the freeze-dried defrost liquid (Table 3-2), where the arsenic concentration was higher in the fried defrost liquid and the lead concentration was higher in the freeze-dried defrost liquid.

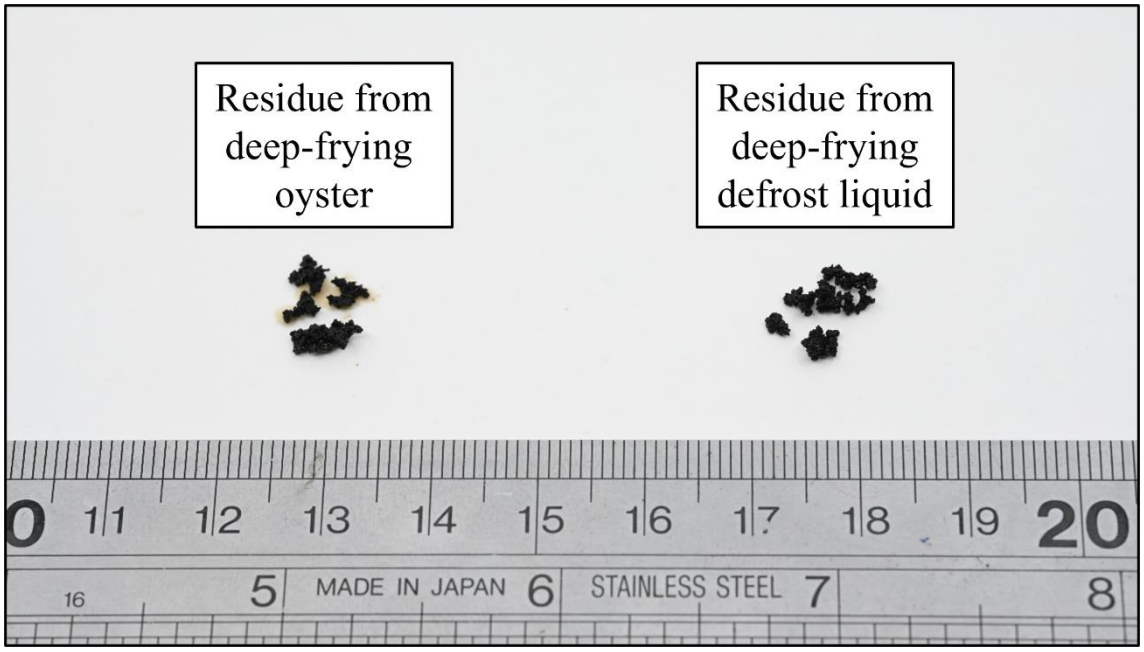


Figure 3-4: Comparison between carbonaceous residues from deep-frying oyster (left) and the residues from deep-frying defrost liquid (right).

3.3.3. Arsenic adsorption study

The second hypothesis on the accumulation of heavy metals in the residues was that the contaminants are first leached into the oil, and they have a strong affinity towards the residues, leading to adsorption of heavy metals onto or into the residues.

The first part of the arsenic adsorption experiment was set up to investigate whether dissolved arsenic and lead would be preferentially adsorbed by residues using deep-frying oil spiked with heavy metals and residues produced by flour mix. The results are summarized in Figure 3-5. No clear deposition of dissolved arsenic into or onto the flour mix residues was observed, as the arsenic concentration of the flour mix residues was not larger than that of the spiked oil. However, there is potential lead adsorption by the flour mix, as the concentration of lead in the flour mix residues is consistently higher than the spiked oil from the same cycle. In particular, the flour mix residues from the first cycle had a lead concentration almost double that of the spiked oil, suggesting possible active adsorption of lead into the flour mix residues.

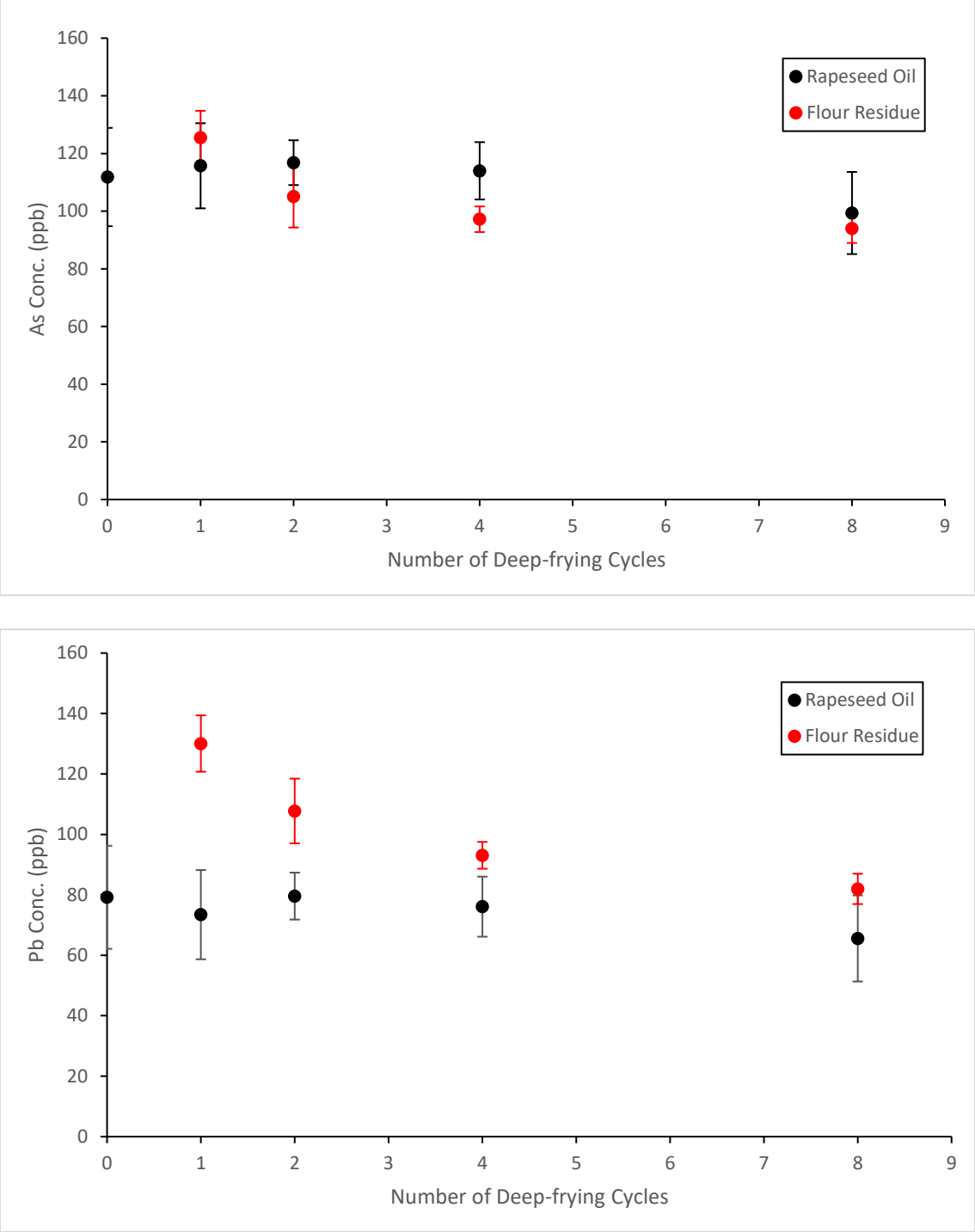


Figure 3-5: Plots of lead concentration (top) and arsenic concentration (bottom) versus number of deep-frying cycles in the arsenic adsorption study using deep-frying oil spiked with arsenic and lead.

The second part of the arsenic adsorption experiment involves using deep-frying oil contaminated with arsenic after repeatedly deep-frying oysters for 64 cycles. In contrast to the first part where arsenic already dissolved in an oil matrix is spiked into fresh deep-frying oil, this experiment uses repeatedly reused oil to see whether there would be any preferential deposition of arsenic onto or into residues when abused oil is used. It was found that the arsenic concentration in the deep-fried batter was 450 ppb versus 412 ppb in the deep-frying oil, suggesting minimal preferential deposition of arsenic onto the batter in a practical setting. Interestingly, carbonaceous residues were observed to be trapped in the batter (Figure 3-6) as the batter mix was deep-fried and the crusts formed. Therefore, this experiment also proved that arsenic accumulated in deep-frying oil can contaminate subsequent batches of food deep-fried, causing the food to have high arsenic content.

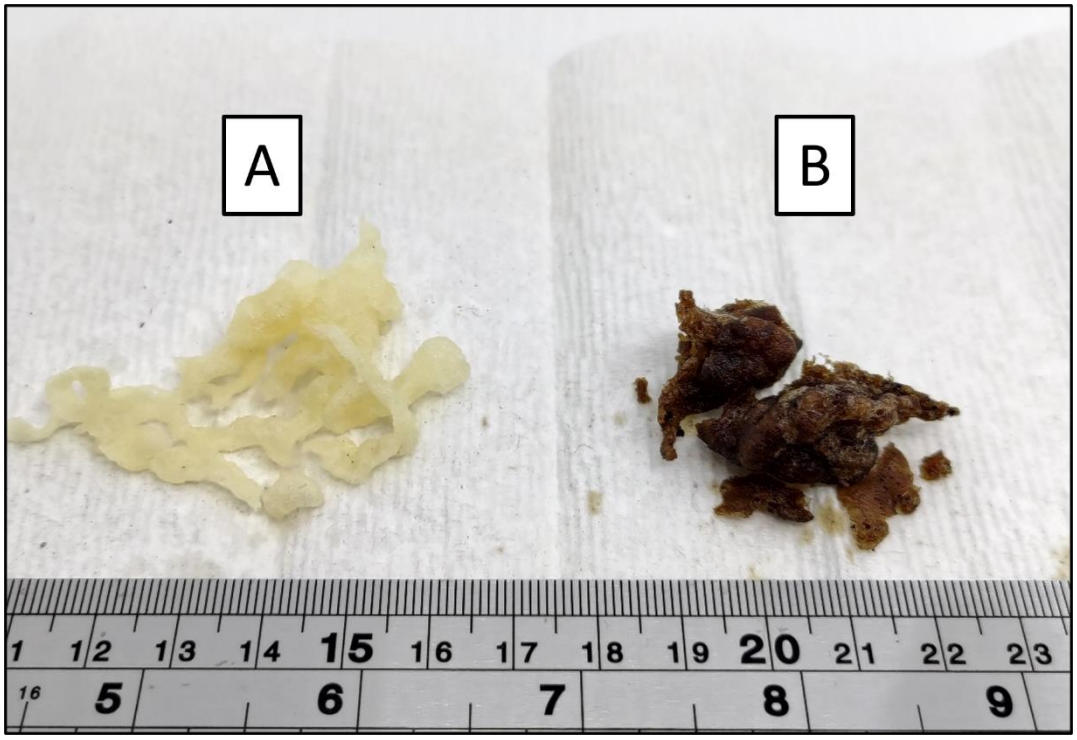


Figure 3-6: Appearance of batter deep-fried in fresh oil (A) versus batter deep-fried in oil used to deep-fry oyster for 64 cycles (B). The colour of the batter from used oil had a lot of residues adsorbed onto the surface, and was much darker than the batter from fresh oil.

3.3.4. Battered food study

According to literature, the use of batter can "act as a barrier against the loss of moisture by protecting the natural juices of foods" during deep-frying[99]. Since the "natural juices" of the oysters leads to formation of residues with enriched heavy metal content, the use of batter should attenuate the accumulation of heavy metals in the deep-frying oil. If this is indeed the case, suggestion on using batter for foodstuff potentially higher in heavy metal content can be included in recommendations published by governing bodies to mitigate the risk of heavy metal accumulation in deep-frying oil.

A comparative study using the same deep-frying parameters as the initial deep-frying experiments without straining was completed and the accumulation trends of the two sets of experiments were compared (Figure 3-7). It was found that the use of batter significantly reduced the accumulation rate of arsenic in deep-frying oil, as the arsenic concentration only reached 134±44 ppb after 64 cycles compared to 567±133 ppb in the no batter experiment, roughly equalling a 75% reduction. Mass loss of the food caused by deep-frying was also compared between the batter set and the no batter set, where the percentage mass loss for the batter set was only 20.7±6.3%, roughly 60% lower than the 52.7±5.3% mass loss with the no batter set (Table S-4). The oysters from the batter set are also visibly more plump compared to the no batter set, suggesting more retained moisture in the battered oysters. The correlation between the mass loss reduction and arsenic concentration reduction is in agreement with the hypothesis that the liquid seeped from the oysters is the main cause of heavy metal accumulation in deep-frying oil.

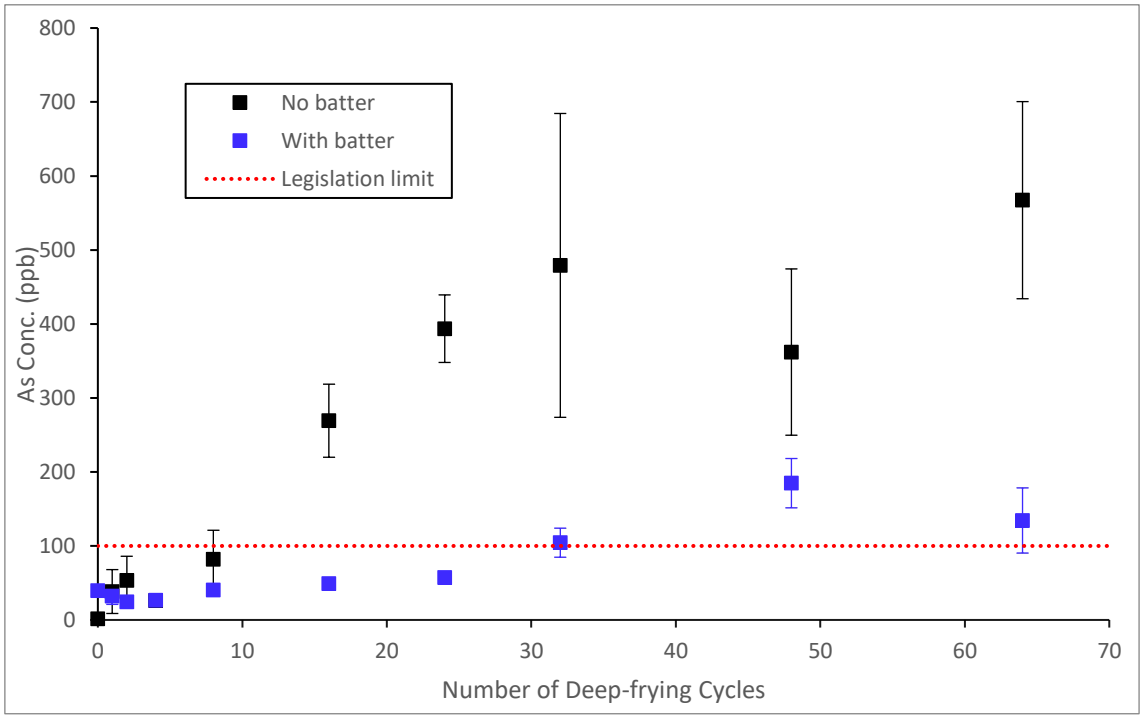


Figure 3-7: Comparison of arsenic concentrations of deep-frying oil with battered food versus without batter.

Results demonstrated that the use of batter effectively shields foodstuff from the oil to prevent liquid from foodstuff to seep into the oil and cause heavy metal accumulation. The use of batter, together with frequent straining of the deep-frying oil, would greatly attenuate the potential heavy metal accumulation issue in deep-frying practices. However, since the heavy metal concentrations were shown to still be able to accumulate to levels above the legislation limit, there may be a need for monitoring heavy metals in deep-frying oil to ensure food safety.

3.4. Conclusions

Arsenic levels of carbonaceous residues found in deep-frying oil were found to be profoundly higher than that of oysters. Two mechanisms for the formation of residues of high heavy metals levels were hypothesized. The first hypothesis was that high levels of heavy metals were trapped inside the residues as they formed from liquid leached from food. The second hypothesis was that the heavy metals leached into the oil were adsorbed by the residues.

Analysis of liquid collected from the defrosting process revealed that the arsenic levels in residues were comparable to dried defrost liquid, suggesting that the first hypothesized mechanism may have led to the enrichment of arsenic in the residues. Meanwhile, the adsorption study demonstrated that there is little preferential deposition of arsenic onto residues. Therefore, the results suggests that the first proposed mechanism was more likely the reason for the buildup of arsenic in residues. Additionally, the adsorption study validated that residues could adhere to batter and lead to arsenic transfer from deep-frying oil containing residues to food deep-fried, demonstrating food safety concerns with residues of high heavy metal content.

Using batter to coat foodstuff before deep-frying can effectively reduce the level of accumulation of heavy metals in oil, and therefore is encouraged in deep-frying practices together with frequent straining of deep-frying oil. However, since the heavy metal concentrations can still be significant and exceed the legislation limit, monitoring of heavy metal content in deep-frying oil may be needed to ensure compliance and more importantly food safety.

Chapter 4:Deep-frying study using other food types

4.1. Introduction

Accumulation of arsenic in deep-frying oil is demonstrated to be possible by the deep-frying experiment using oysters in Chapter 2. To investigate whether accumulation of arsenic is also possible using other types of food, other food types including chicken and fish were selected for comparison because of their popularity among consumers.

Deep-fried chicken, including wings, breasts, drumsticks, thighs, nuggets and others, is one of the most popular types of deep-fried food. The market size of fast-food chicken industry saw an increase of 9.4% to 55 billion USD in 2022[100], illustrating that the market is huge and rapidly growing. Deep-fried chicken was also one of the most studied type of deep-fried food, with a lot of well-cited articles focusing on different aspects of the deep-frying process using chicken as a model[101-105]. Arsenic contamination of chicken meat due to the use of arsenic-based drugs in poultry production was a significant concern in multiple parts of the world in the past[106, 107], and thus deep-frying of chicken is included in this study despite recent report of low arsenic levels in chicken meat and associated products in Hong Kong[108].

Deep-fried fish is also one of the more popular types of deep-fried food. One well-known example of deep-fried fish is the iconic dish fish and chip, which is claimed to be the most popular takeaway meal choice in Britain[109]. Besides the fish meat, low value fish products such as fish frames, fish skin and fish roe and by-products such as fish cakes and fish balls can involve deep-frying processing as well[110]. Fish can contain higher levels of heavy metals, especially arsenic and mercury, depending on the species and thus is considered major dietary sources of arsenic and mercury to humans[111]. A previous study

by Burger et al. [112] has demonstrated that mercury concentration in deep-fried fish can increase relative to raw fish, and the increase in concentration can lead to overestimation of safe consumption amounts and in turn cause intake to exceed acceptable levels. Therefore, fish is included in this study to evaluate the food safety risks associated with deep-frying fish; grouper fish fillet was selected for its relatively high arsenic content as reported by the CFS[108].

Lastly, oysters from another origin were also tested to check the reproducibility of the experiment if different oysters were used. The heavy metal concentrations in oysters can vary depending on the origin among other factors, and thus oysters from another origin were chosen to see if the results would be similar.

4.2. Experimental

4.2.1. Materials and chemicals

Kronfågel frozen chicken mid joint wings from Sweden, Sun Marine frozen grouper fish fillets from Vietnam, and Sun Marine frozen oysters from Korea were purchased from local supermarkets (Figure 4-1) and used in this experiment. The defrost liquid of the Korean oyster and the grouper fish fillet were collected using the same method described in Chapter 3.2.3. The rapeseed oil used was Hop Hing (合興) SS Rapeseed Oil (SS 菜籽油) purchased from Ball Kee grocery store (波記糧食), same as the oyster experiments for direct comparison. The methods and chemicals used for analysis, including the microwave digestion and the total arsenic instrumental analysis, were the same as described in Chapter 2.2.

Figure 4-1: Photos of frozen chicken mid joint wings (A), frozen grouper fish fillets (B), and frozen Korean oysters (C) used in this study.

4.2.2. Sample preparation and deep-frying operation

Stock solutions and foodstuffs were stored according to suppliers' instructions. The deep-frying parameters used such as the deep-frying time, temperature, food to oil ratio, and approximate weight of food were the same as that of the oyster experiments for better comparison. The total arsenic and lead limits of 100 ppm in edible oils in the Food Adulteration (Metallic Contamination) Regulations in Hong Kong was again used as a reference of significant arsenic accumulation. Filter paper used for the collection of residues in deep-frying oil was GC-50 grade glass fiber membrane filters produced by Advantec (Tokyo, Japan).

4.3. Results and discussion

4.3.1. General observations

Change in the quality of deep-frying oil was observed in all sets of experiment, but the degrees of deterioration were very different. The changes in deep-frying oil colour were similar for the chicken wing and grouper fish fillet set and were both significantly slower compared to the rapid changes seen in oyster sets, where the Japanese and Korean sets exhibited similar degrees of colour change (Figure 4-2). This was reflected in the colour of the food yielded, as the change in the colour of the deep-fried oysters were much more rapid compared to that of the chicken wings and the fish fillets.

An interesting point to note was that both the deep-frying oils of grouper fish fillet and chicken wing sets exhibited some degree of cloudiness in samples collected in later cycles, suggesting that there are possibly suspended fine residues in the oil that settle at a slower rate. This notion was supported by the sediment of residues after allowing the oil to rest for prolonged periods of time, giving clearer oils (Figure 4-2).

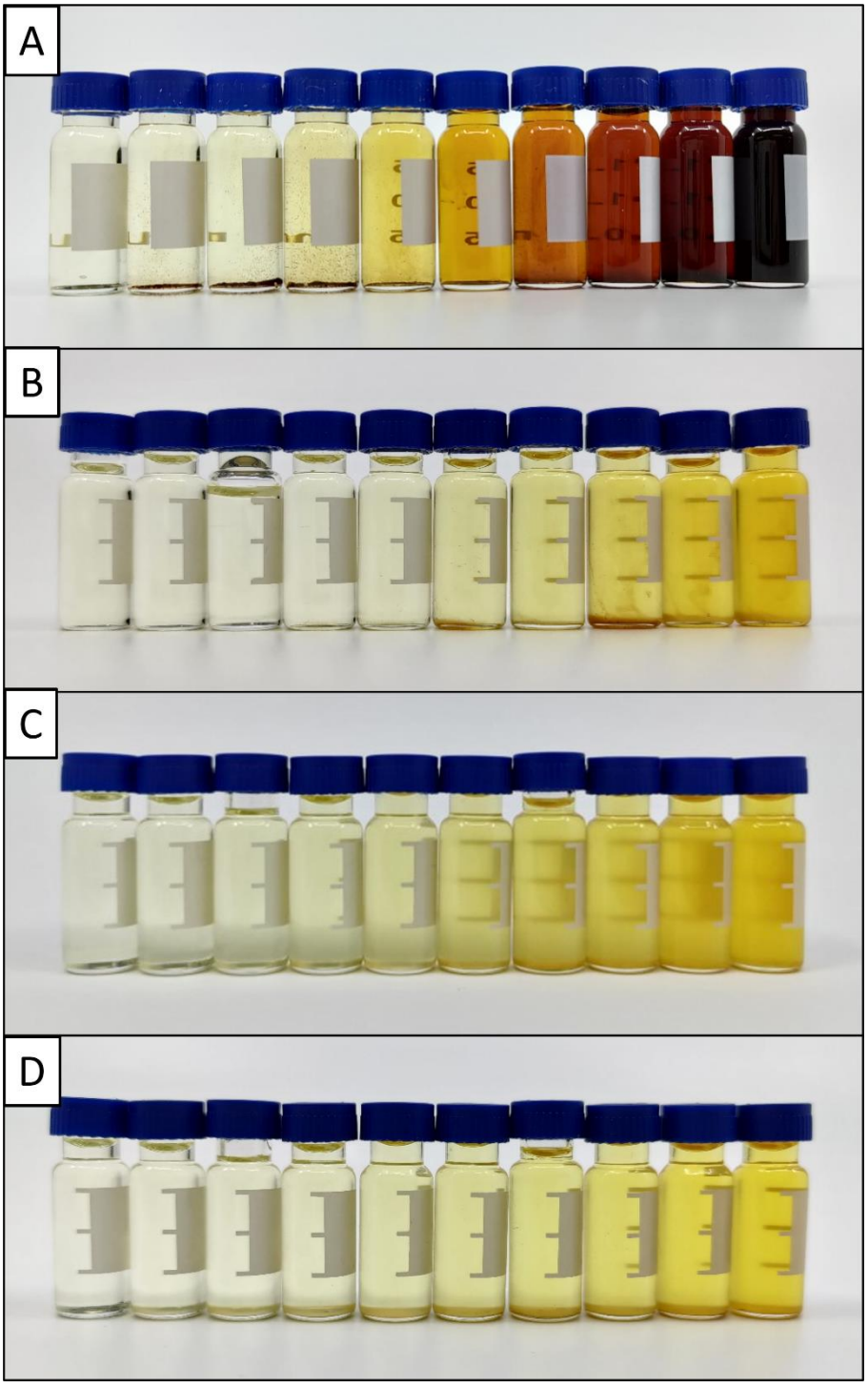


Figure 4-2: Colour change of oil used to deep-fry (A) Korean oyster, (B) Chicken wings, and (C) grouper fish fillet over 64 deep-frying cycles, and the same samples from grouper fish fillet after allowing to settle for 48 hours (D). The cycle numbers of the samples from left to right are 0, 1, 2, 4, 8, 16, 24, 32, 48, and 64.

The observation of suspended fine residues in oil was similar to that described in Chapter 2.3.3., but more noticeable because cloudiness in deep-frying oils from oyster sets would be difficult to observe due to the much darker colours of the deep-frying oils. The residues contributing to cloudiness in the deep-frying samples seemed to be finer for the grouper fish fillet set compared to the other sets, as more significant sedimentation can be observed for both the Korean oyster set and the chicken wing set without allowing the samples to settle. This is possibly due to the difference in composition of the liquid leached from the different types of meat.

TPC, a more quantitative method, was also employed to assess the quality of the deep-frying oils. After 64 deep-frying cycles, the deep-frying oil of the Korean oyster set, much like that of the Japanese oyster set, reached a TPC value beyond the handheld TPC device detection limit of 40%; whereas the deep-frying oil of the chicken wing and grouper fish fillet set only reached 28% and 29% respectively after 64 cycles. Both results indicate that the oil deterioration rates were much higher with oysters compared with either the chicken or fish.

The difference in oil deterioration rates was possibly due to the combined effect of the difference in mass transferred from food to oil and the difference in moisture content. This was evident when examining the comparison of mass losses during deep-frying for different food types. For example, the percentage mass loss for Japanese oysters during deep-frying, calculated by dividing the mass loss by the mass before deep-frying, approximately ranged from 40-60% with an average of 51%; whereas for the grouper fish fillet the percentage mass loss approximately ranged from 30%-45% with an average of 36%. This mass loss difference would contribute not only to the mass transferred from

food to oil, but also the amount of moisture exposed to the deep-frying oil. As moisture in deep-frying oil is critical to hydrolysis reactions in deep-frying oil deterioration, increased moisture exposure can lead to accelerated deterioration.

It was observed during deep-frying that the abundance of carbonaceous residues seen when deep-frying oysters was not seen with chicken wing or grouper fish fillet. Deep-frying chicken wings yielded some filterable residues but not as much as that of the oyster sets. Deep-frying grouper fish fillets yield a very small amount of very fine, orangish brown residues (Figure 4-3) that could not be strained using the same strainer used to remove residues from oil used to deep-fry oysters. Since the residues from grouper fish fillets were too fine to be collected using the strainer, it was only collected after all 64 deep-frying cycles by filtration of the deep-frying oil using glass fiber membrane filters. Notable differences between the residues yielded from grouper fish fillets and residues yielded from oysters besides the residues size include the difference in colour (Figure 4-3) and the total amount of residues yielded: the obtained amount of residues from 64 cycles of deep-frying grouper fish fillets was only approximately 1 gram, which was very little compared to the oyster sets which yielded over 20 grams of residues in total.

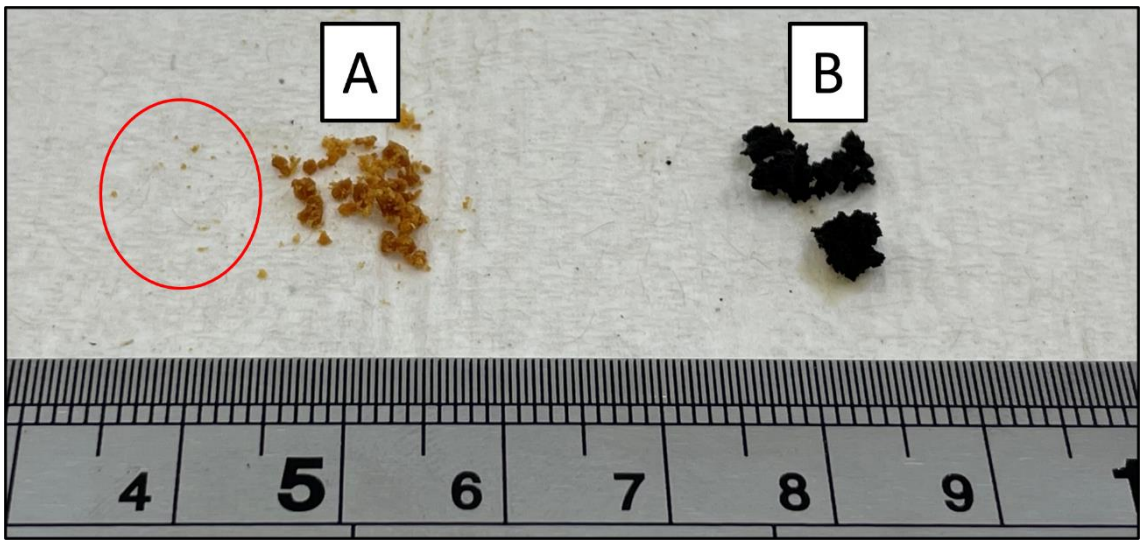


Figure 4-3: Appearance of residues from grouper fish fillet (A) versus residues from oysters (B). Residues from grouper fish fillet are clumped up after filtration, but particles much finer than that of the oysters can still be observed scattered around the pile (red circle). The contrast in colour should also be noted.

4.3.2. Elemental analysis

All sets of deep-frying experiments did not result in significant accumulation of lead, and other possible heavy metals were again scanned and did not demonstrate accumulation tendencies. Results from the chicken wing set demonstrated there is minimal associated food risk, as the raw chicken, cooked chicken and the deep-frying oil used did not contain detectable levels of arsenic. In contrast, the Korean oyster set further demonstrated arsenic accumulation is possible using oysters. Both the raw oysters and the defrost liquid had similar arsenic levels to that of Japanese oysters, and the arsenic accumulation trend in deep-frying oil closely resembled the trend seen in the Japanese oyster set. A comparison between the data collected from the chicken wing, Korean oyster and Japanese oyster sets can be seen in Figure 4-4.

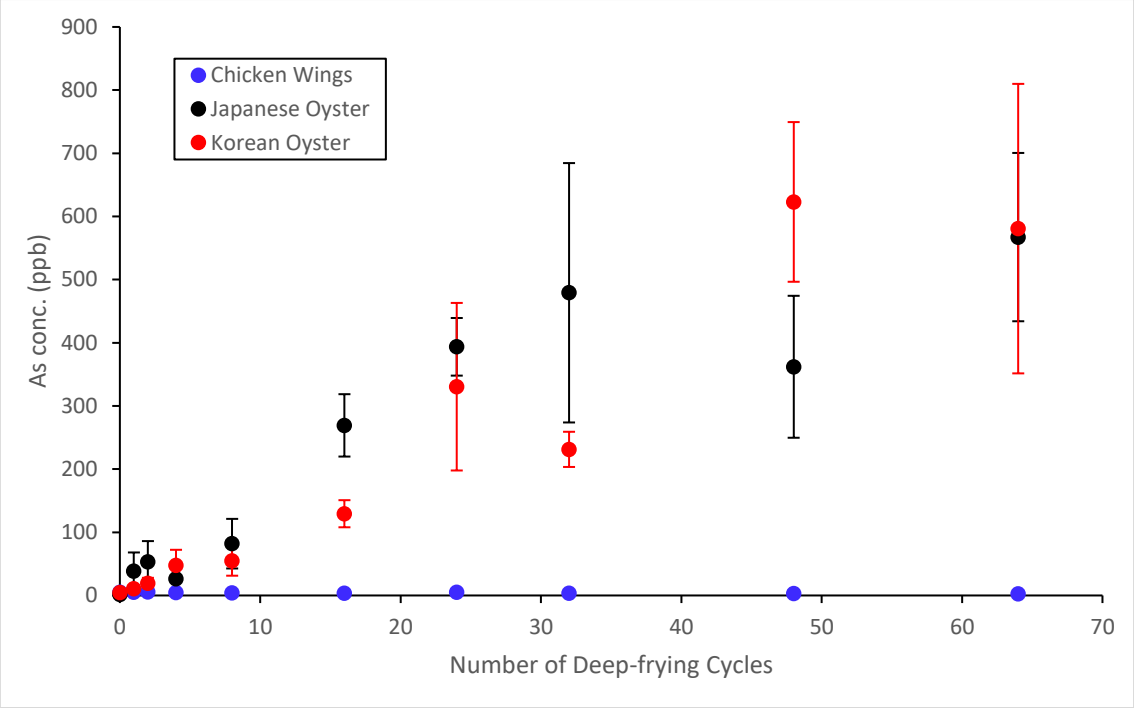


Figure 4-4: Arsenic concentrations versus number of deep-frying cycles for the chicken wing and Korean oyster sets versus the Japanese oyster set.

Of the 3 sets of experiment, total arsenic analysis of samples from the grouper fish fillet set gave the most interesting results. Arsenic levels of raw grouper fish fillets (2.66 ppm) were comparable to that of raw Japanese oysters (2.44 ppm), and a similar observation was made for the defrost liquids (0.775 ppm for grouper fish fillet versus 1.51 ppm for Japanese oyster) (Table 4-1). This resulted in arsenic accumulation in the deep-frying oil in the grouper fish fillet set, at a rate and to a degree similar to that of the oyster sets (Figure 4-5). Combine this with the slower deterioration of the deep-frying oil from the grouper fish fillet set, the risk of food trade unknowingly violating the regulations as well as the food safety risk is much higher with the grouper fish fillet. If the deterioration is slow, the deep-frying oil may not be replaced in time and lead to significant arsenic accumulation. Whether the user replace the oil by colour or by TPC value, the

replacement of oil would be much later in the case of grouper fish fillet compared to the case of oysters while the arsenic builds up at a similar pace.

Table 4-1: Arsenic concentration of meat and defrost liquid comparison between Japanese oyster, Korean oyster, and grouper fish fillet.

	Arsenic concentration in meat (ppm)	Arsenic concentration in defrost liquid (ppm)
Japanese oyster	2533±27	1507±46
Korean oyster	2089±173	1241±26
Grouper fish fillet	2659±124	774.9±262.5

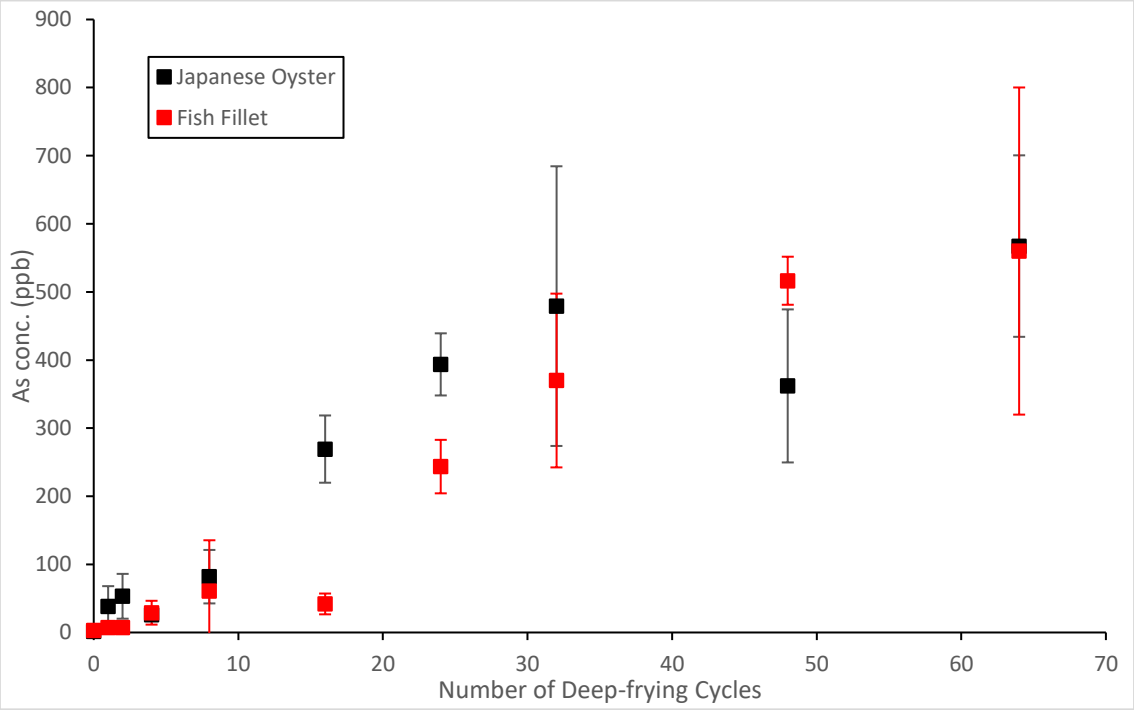


Figure 4-5: Arsenic concentrations versus number of deep-frying cycles for the grouper fish fillet set versus the Japanese oyster set.

Potential buildup of other elements was again studied using IntelliQuant. elements that were of high levels in the raw grouper fish fillets were also accumulated in the oil (Figure 4-6), much like the case of oysters. Some elements that were of higher concentrations in raw oysters, such as copper and silver, demonstrated tendency to accumulate with the oyster set but did not accumulate when deep-frying grouper fish fillets, as they were of low concentrations in the fish. This again supported the hypothesis that only elements that is rich in the food deep-fried would be accumulated in the oil.

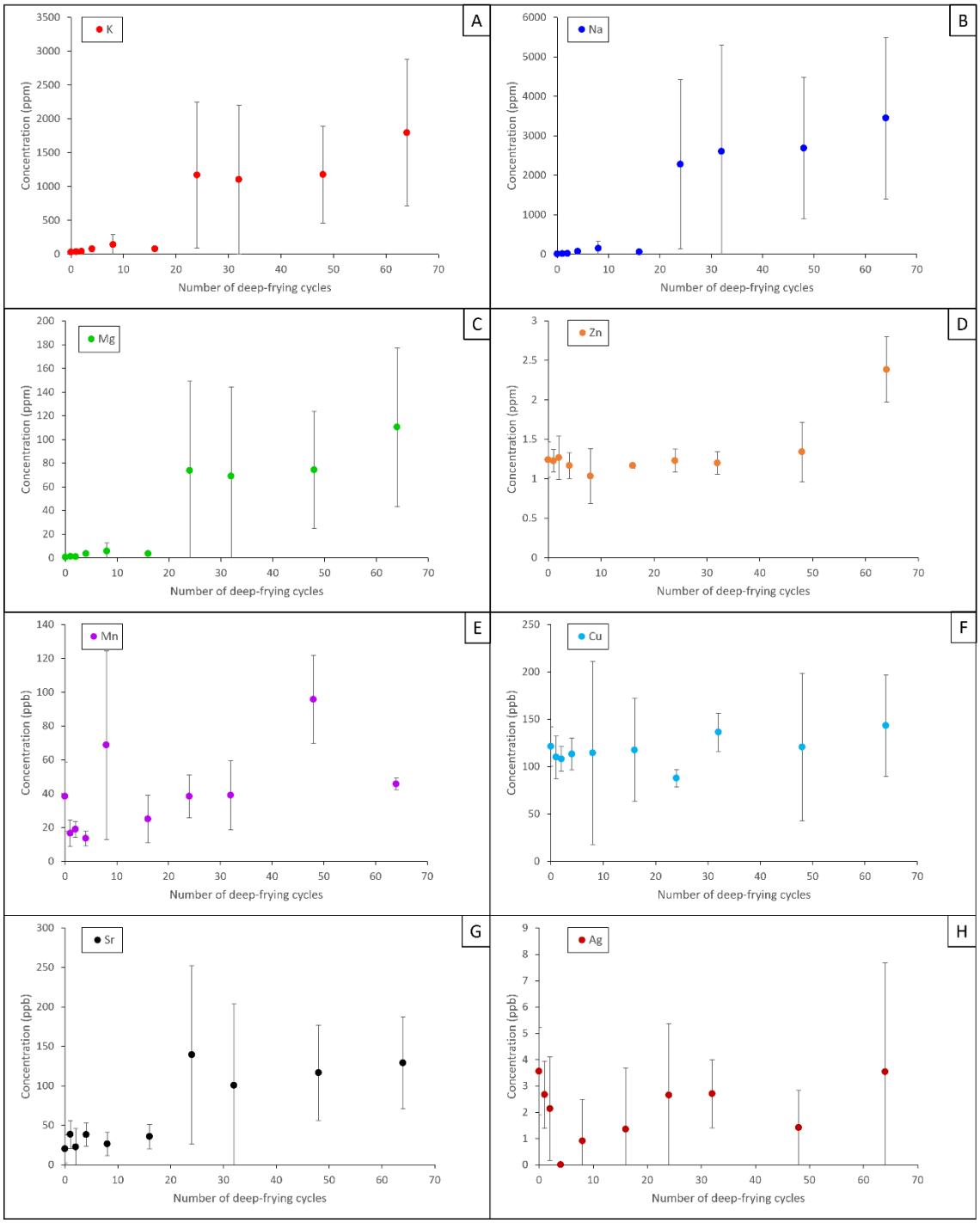


Figure 4-6: Concentrations of various elements in deep-frying oil versus number of cycles of deep-frying grouper fish fillet.

Residues yielded from deep-frying grouper fish fillet, similar to residues from the oyster set, contained high levels of arsenic. However, the arsenic concentration in the residues

from grouper fish fillet was at approximately 26 ppm, which is about 4.4 times higher than the average arsenic concentration of residues from oyster at 5.9 ppm. The difference in concentration is proposed to be caused by the higher moisture content of liquid coming from grouper fish fillet meat compared to that of oysters. Moisture was evaporated as the liquid from the grouper fish fillet or the oyster came into contact with the deep-frying oil, leaving dried residues suspended or settled in the pan of oil. During this process, the arsenic was essentially enriched, giving residues of high arsenic concentration. Since the moisture content of the liquid from grouper fish fillet was higher than that of oyster (98% versus 90%), the enrichment effect was more significant, giving residues of higher arsenic concentration.

The arsenic adsorption study reported in Chapter 3.3.3 demonstrated residues of high arsenic concentrations may be adsorbed by food subsequently deep-fried in the same pan of oil, causing unwanted and unknowing intake of arsenic. The colour of the residues yielded from deep-frying grouper fish fillet was significantly lighter compared to the residues yielded from oysters, and therefore any adsorption onto the surface subsequently deep-fried food would not be as noticeable as the case of residues from oyster shown in Figure 4-3. The orangish brown colour, much lighter than the colour of residues from oysters, would blend in well with the typical colour of the crust of deep-fried food, leading to underestimation of associated food risk.

Further studies on the change of arsenic species are needed to fully evaluate the associated risks, but it is very likely that the residues from grouper fish fillet would have a similar distribution of arsenic species compared to that of oysters. This is because much like in oysters the majority of arsenic in fish muscle is present as AsB, where the percentage can

be up to 90%[113]. The AsB in the residues from grouper fish fillet should be prone to transform to a more toxic species under deep-frying conditions.

4.4. Conclusions

Arsenic accumulation in deep-frying oil was significant when grouper fish fillet is deep-fried, reaching levels comparable to deep-frying oils used to deep-fry oysters. Yet, the deep-frying oil quality of the oil from the grouper fish fillet set was remarkably higher than that of the oyster sets, as demonstrated by the lighter colour and lower TPC value of the deep-frying oil of the grouper fish fillet set. The implication of the difference in deep-frying oil quality is that oil used to deep-fry fish may not be replaced as frequently but the arsenic accumulation is no less rapid compared to oil used to deep-fry oysters, and thus would pose an even greater risk both in terms of food safety and of unknowingly violating the regulations in Hong Kong. The high arsenic concentration together with the relatively light colour of residues from grouper fish fillet are of particularly high concern, as it would be less noticeable when adhered onto food that was subsequently deep-fried. Overall, the lighter colour would increase the likelihood of consumption of food of high arsenic content and is therefore alarming.

Chapter 5:Arsenic speciation study

5.1. Introduction

5.1.1. ICP-MS and ESI-MS in arsenic speciation

Toxicity of arsenic can vary greatly between species, and thus speciation of arsenic in food is particularly important for food safety risk assessment. Extraction, analysis, and identification of arsenicals are steps in speciation, and are therefore considered a focus of this study.

Extraction of arsenicals is typically done by mechanical mixing, sonication or microwave-assisted extraction. Recoveries of the extraction step can be affected by factors including the matrix, species present, solvents used, and extraction method, time and temperature[114], and tedious studies may be required for the development and validation of the extraction method. Previously reported extraction method was used in this study and will be discussed later in the experimental section.

Analysis and identification of arsenicals can be even more challenging, especially since in this study unknown arsenicals may form under the extreme conditions of high heat and hydrophobic environment. Liquid chromatography-inductively coupled plasma-mass spectrometry (LC-ICP-MS) and liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) are the most commonly used analytical techniques for arsenic speciation, where LC-ICP-MS provides element-specific and easily quantifiable results and LC-ESI-MS provides complementary information that allows structural identification.

Electrospray ionization mass spectrometry (ESI-MS) is the most used method for complementing ICP-MS in arsenic speciation analysis. Compared to the element-specific ICP-MS, ESI-MS is considered to be species-specific[115], or in other terms compound-specific[116]. It provides structural information of the molecule since the injection is not atomized and the molecular structure is preserved. ESI-MS on its own has significant limitations: it is not element specific, some arsenicals are difficult to ionize, and co-eluting matrices and organics can interfere with ionization and fragmentation. However, ESI-MS is an excellent tool for obtaining species specific information and enable the identification of unknown species as molecular information is retained. Ionization of sample is achieved using an electrospray, which is the aerosolization of liquid by applying a voltage (Figure 5-1). This technique is referred to as a soft ionization technique because it produces molecular ions with little tendency to fragmentate.

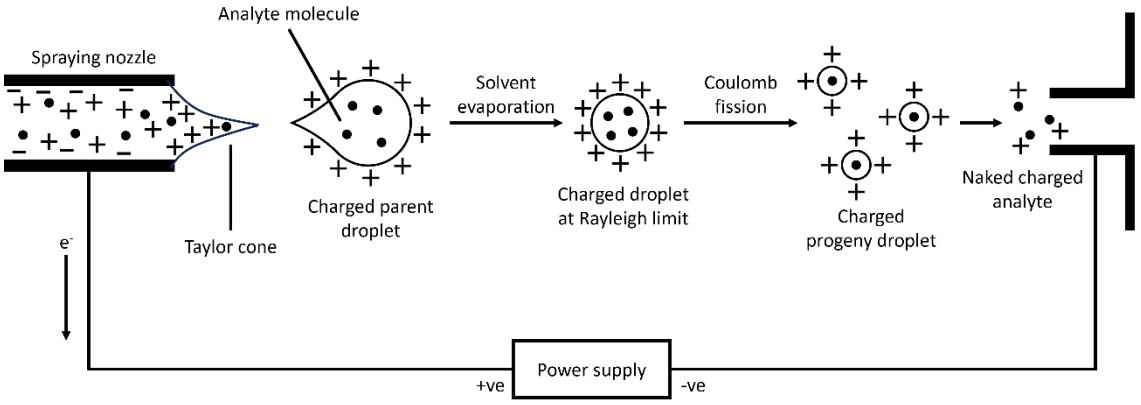
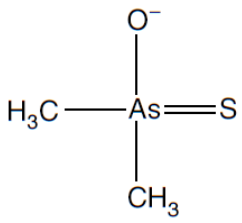
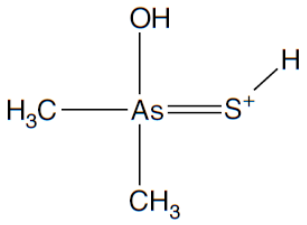
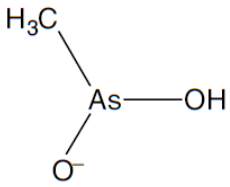
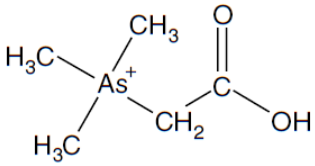


Figure 5-1: Graphical illustration of ESI[117].

An interesting point to note is that ESI can produce ions in either the positive mode and the negative mode, and the use of both modes may be required for the identification of all arsenicals of interest. Some arsenicals, such as dimethylmonothioarsinic acid ($DMMTA^{V}$), can be detected in both the positive and negative modes while others such as MMA^{III} (negative mode) and AsB (positive mode) were only reported in one of the

two modes (Table 5-1). Therefore, both positive mode and negative mode should be used for thorough analysis of arsenicals.

Table 5-1: Example of arsenicals detected in both positive and negative mode and only in either mode[118].

Name	Polarity	Molecular ion structure	Molecular ion *m/z*
$DMMTA^{V}$	Negative		153
$DMMTA^{V}$	Positive		155
MMA^{III}	Negative		123
AsB	Positive		179

Detection of fragmentation peaks using tandem mass spectrometry, often referred to as MS/MS, is the next crucial step in arsenical identification. Tandem mass spectrometry is the utilization of at least two stages of MS analysis: the first stage selects ions of a particular *m/z,* and the subsequent stage identifies the selected ions by examining the *m/z* of ions fragmented from the starting ions (Figure 5-2). The ion selected in the first stage are conventionally called the precursor ion, and the fragments product ions. Ways to produce fragment ions include surface-induced dissociation, absorption of

electromagnetic radiations, electron-capture dissociation, and most commonly collision-induced dissociation[119].

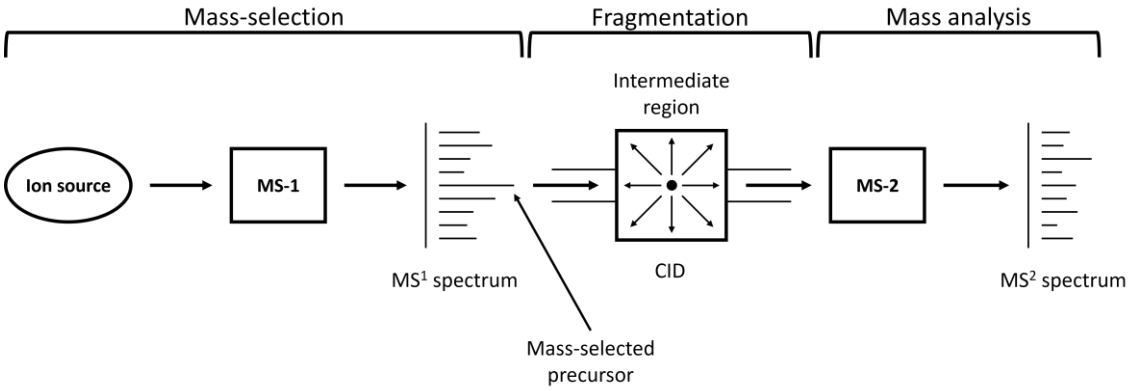


Figure 5-2: Graphical illustration of the principle of tandem mass spectrometry[119].

Tandem MS has three main types of scan functions: product-ion scan, precursor-ion scan, and neutral-loss scan[120]. The first two are more commonly used in the identification of arsenicals and thus is focused upon here. The product-ion scan is a scan with the precursor ion fixed at a *m/z*. This function is effective when one knows the accurate mass of the precursor ion and wants to deduce its identity by studying the product ions formed after dissociation. As for the precursor-ion scan, it is a scan that search for all precursor ions that may produce fragments of a specific *m/z*. This is particularly useful for the identification of unknown arsenicals, as one can set the product ion *m/z* at 74.92 to search for precursor ions that fragment to give arsenic as a product ion. However, the sensitivity of ESI-MS is typically lower than ICP-MS; unwanted peaks and noise can often mask the targets.

Due to the limitations of each technique, ICP-MS and ESI-MS are often paired together in arsenic speciation analysis. Complementation of ICP-MS with ESI-MS is done by

matching peaks from ICP-MS to peaks from ESI-MS, allowing easy identification of peaks in ESI-MS that contain arsenic by matching element-specific peaks from ICP-MS. This complementation can either be done by "online" analysis, which is simultaneous ICP-MS and ESI-MS analyses by splitting the flow out of LC (Figure 5-3), or "offline" analysis, which is comparing retention times of eluted peaks to allow identification and quantification. Online analysis (also called parallel analysis) is more intuitive, as ICP-MS and ESI-MS are done simultaneously. Typically, a larger portion of the flow out of LC (75-90%) was fed to the ESI-MS while the rest was fed into ICP-MS because of the higher sensitivity of the ICP-MS. Offline analysis, to compare, enables identification by using similar chromatographic parameters for direct comparison of retention times between ICP-MS and ESI-MS data. Due to limitations in resources available, the offline method will be used in this study.

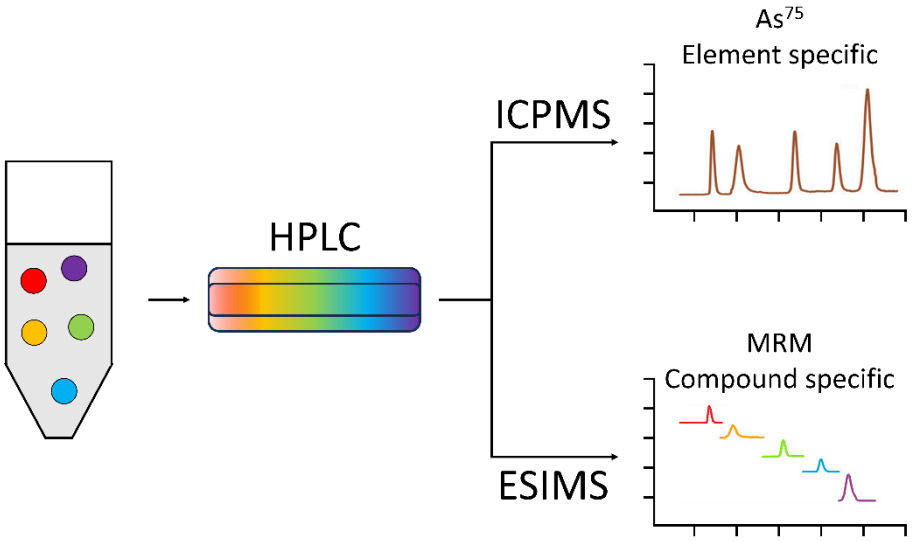


Figure 5-3: Parallel ICP-MS and ESI-MS analysis[116]. HPLC stands for high-performance liquid chromatography. Parallel analysis, or called split-flow analysis, allow easy and confident assignment of peaks, but peak assignment can still be done if ESI-MS and ICP-MS are done separately.

A recent demonstration of the robustness of the complementary use of ICP-MS and ESI-MS was the study by Peng et al. [116] where chicken liver samples were analysed to study possible metabolic pathways of 3-nitro-4-hydroxyphenylarsonic acid (roxarsone, ROX), a feed addictive containing arsenic that is sometimes used in poultry production. Liver samples of chicken fed either with a controlled diet or ROX-supplemented diet were analysed using HPLC-ICP-MS. Not surprisingly, both the amount and number of species of arsenic were higher in the ROX chicken liver sample, and a total of 11 peaks were seen in the ROX chicken sample. 3 of the peaks did not match any arsenic standards the research team had. To further investigate the unknown species, an online ICP-MS/ESI-MS approach was employed. To identify the unknown metabolites, the authors applied a precursor ion scan method using characteristic fragment ions for arsenicals: AsO^- (*m/z* 91), AsO^{2-} (*m/z* 107), CH^2AsO^{2-} (*m/z* 121), and AsO^{3-} (*m/z* 123). One of the unknown peaks was preliminarily identified as methyl-ROX using this method (Figure 5-4). The identification was further confirmed as high-resolution time-of-flight mass spectrometry gave an accurate *m/z* of within 0.8 ppm of the theoretical *m/z* for methyl-ROX, and the fragment ions observed match expected fragment ions for the structure of methyl-ROX. The last step of the confirmation was to synthesize methyl-ROX and used it for analysis and spiked samples, where the peak retention time and fragmentation ions both matched the unknown peak. The two other unknown arsenic compounds were identified to be methylated 3-amino-4-hydroxyphenylarsonic acid and methylated 3-acetamido-4-hydroxyphenylarsonic acid using the same strategy.

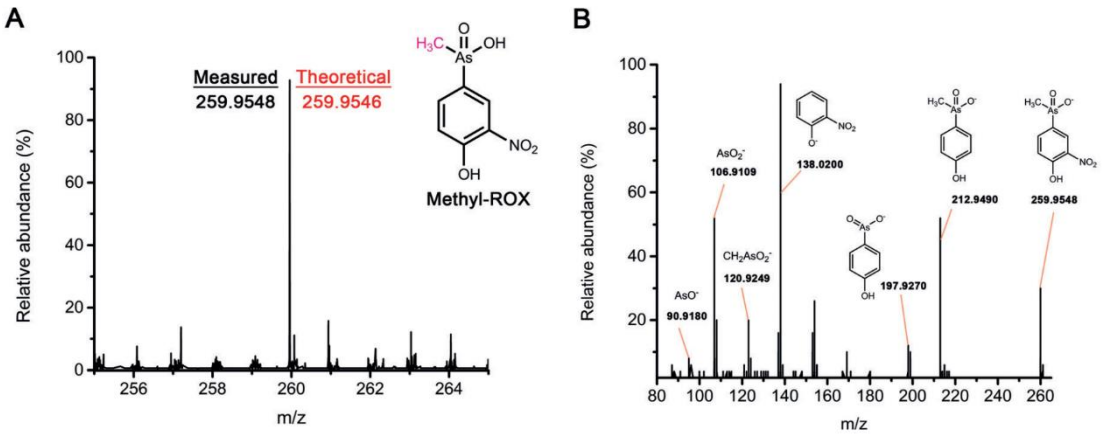


Figure 5-4: Identification of newly discovered Methyl-ROX using ESI-TOF-MS analysis[116]. Evidence to support the identification included (A) the accurate mass of the peak at *m/z* 259.9548 and (B) the product ion spectrum of *m/z* 259.9548 showing the specific fragment peaks.

5.1.2. Arsenical transformation under various conditions

As mentioned in Chapter 1, predominant arsenic species found in seafood include MMA, DMA, AsB, AsC, TMAO and TMA^+, and typically the amount of more toxic inorganic arsenic is low. However, some researchers suggest changes in concentrations of species of arsenic under various conditions. Devesa et al[26] claimed that cooking can increase the concentration of inorganic arsenic in bivalves and squid, and AsB can change to more toxic TMA^+ ion in fish. However, in a conflicting study by Dahl et al[11], it was claimed that the concentration of inorganic arsenic did not change significantly upon boiling, frying or storage by freezing (but concentrations of organic species can change upon being processed). In a study by Ichikawa et al[34] it was shown that the inorganic arsenic level, specifically arsenate, in Hijiki algae is significantly reduced when treated by a Japanese cooking method that involves soaking the algae in water followed by cooking. Among the mentioned studies, the study by Devesa et al[26] has the strongest resemblance to deep-frying in terms of cooking temperature; but it should be noted that the conditions of deep-frying is without doubt harsher as a number of undesirable conditions can be introduced by poor practice, such as overheating the oil, not replacing the oil, or leaving food debris and residues in hot oil for prolonged periods of time compared to the short cooking time used in the study by Devesa et al[26]. The major arsenic species in oysters being AsB^{121} also increases the comparability between this study and the study by Devesa et al. Thus, the risk of arsenicals transforming into more toxic species is probable for deep-frying practices.

In this study, carbonaceous residue can be continuously heated in the oil for hours if unfiltered. Therefore, this part of the study should be able to help identify the potential

health risks associated with arsenic in deep-frying of seafood products. This study focused on samples obtained from the deep-frying experiments using oyster.

5.2. Experimental

5.2.1. Materials and chemicals

Arsenic standard solutions used include iAs^{V}, iAs^{III}, DMA and AsB solutions. iAs^{V}, iAs^{III} and DMA 10000 ppm stock solution were prepared from sodium arsenate dibasic heptahydrate, sodium (meta)arsenite and sodium cacodylate trihydrate produced by Sigma-Aldrich (St. Louis, Texas) respectively. AsB solution was purchased as arsenobetaine purum from Sigma-Aldrich (St. Louis, Texas). Working 4 standard mix solutions were prepared on the day of analysis to ensure minimal change between species.

Ammonium bicarbonate (BioUltra, ≥99.5%) (NH_4HCO_3) and ammonium dihydrogen phosphate (99.999% trace metals basis) ($NH_4H_2PO_4$) used for liquid chromatography were produced by Sigma-Aldrich (St. Louis, Texas). Methanol with 0.1% formic acid used for LC-ESI-MS analyses was prepared with LC-MS grade methanol produced by Anaqua Chemicals Supply (Wilmington, Delaware) and formic acid produced by Sigma-Aldrich (St. Louis, Texas).

5.2.2. Arsenical extraction

One of the most commonly used extraction method is sonication-assisted extraction using a 1:1 methanol/water mixture (v/v), allowing the extraction of arsenicals from algae, crustaceans, fish, molluscs, terrestrial plants and others with satisfactory recoveries[28]. In this study, the extraction method by Zhang et al. [122] was followed with slight modifications.

For each extraction, about 0.5 g sample was weighted into a 50 mL centrifuge tube. 10 mL of 1:1 methanol/water mixture (v/v) was added to the centrifuge tube. The sample was sonicated for 30 minutes and then centrifuged for 5 minutes at 10,000 rpm, yielding supernatant that was collected. The extraction process was repeated two more times and the supernatant was combined into a flat-bottomed flask. The combined extract of approximately 30 mL was enriched by rotary evaporation at 40 C until a volume of less than 10 mL was reached. The extract was then freeze-dried and reconstituted to 10 mL for instrumental analysis.

5.2.3. Instrumental analysis

Chromatographic analysis of LC-ICP-MS was carried out using an Agilent 1260 Infinity II LC system equipped with a Hamilton PRP-X100 HPLC column (4.1 mm × 25 cm, 10 μm), an auto-sampler and a quaternary solvent system.

In the first trial, an isocratic method using 30 mM NH_4HCO_3 adjusted to pH 8.5 was used. Injections were set for 28 minutes. In the second trial, a gradient method using (A) ultrapure water and (B) 60 mM NH_4HCO_3 was used. The run started with 90% of the A solvent and 10% of the B solvent until 12 minutes, then slowly increased to 100% B until 25 minutes, lastly returning to the initial composition at 26 minutes. A 2-minute window between injections was allowed between runs for flushing.

In the binary solvent method, the mobile phase consisted of (A) 60mM NH_4HCO_3 and (B) 60 mM $NH_4H_2PO_4$ and the gradient elution program was 0% (B) at 0-3 min; 0-100% (B) at 3-14 min; 100-0% (B) at 14-15 min, with a 2-minute window between injections for allowing the column to be sufficiently flushed with the starting eluent. The flow rate was set at 1 mL/min and the injection aliquots were set at 50 μL.

In the final method, the mobile phase consisted of (A) milliQ water and (B) 60 mM $NH_4H_2PO_4$ adjusted to pH 8.75 and the gradient elution program was 5% (B) at 0-8 min; 5-100% (B) at 8-9 min; 100% (B) at 9-18 min; 100-5% (B) at 18-19 min and held at 5% until 20 min, with a 2-minute window between injections for allowing the column to be sufficiently flushed with the starting eluent. The flow rate was set at 1 mL/min and the injection aliquots were set at 50 μL.

The ICP-MS settings used were the same as described in Chapter 2.2.4.

LC-ESI-MS analyses were conducted using the Agilent 6540 Liquid chromatography - Electrospray Ionization Quadrupole-Time-of-Flight Mass Spectrometer (LC-ESI-QTOF).

The mobile phase composition used for LC was modified from the composition used for the LC-ICP-MS to include organic solvent because the inclusion of organic solvent in ESI would greatly improve the signal intensity[123]. NH_4HCO_3 instead of $NH_4H_2PO_4$ was chosen as the mobile phase in conjunction with methanol due to the volatility of the salt enabling high signal intensity[124]. 60 mM NH_4HCO_3 and methanol with 0.1% formic acid at a ratio of 80:20 was used as the mobile phase.

5.3. Results and discussion

5.3.1. Liquid chromatography parameters optimization

Resolution of arsenicals using liquid chromatography has long been a focus in studies related to arsenic due to the difficulties in achieving satisfactory resolution. Summaries written by reviewers[28, 125, 126] described the variety of column types and mobile phases used in attempt to achieve efficient resolution. Under neutral pH, iAs^{III} carries no charge while iAs^{V}, DMA and MMA exist as anionic species, making resolution using anion exchange chromatography optimal[125]. Therefore, one of the most used columns is the Hamilton PRP-X100 anion exchange column (Figure 5-5) for the effective resolution of the arsenicals of highest toxicological concerns. It can enable the resolution of at least 14 different arsenicals[28], making it ideal for identification of as many arsenicals as possible in one run. A very similar product, the Hamilton PRP-X110 provides lower limits of detection with similar selectivity by utilizing a lower total exchange capacity[127]. However, lowered exchanged capacity would lead to poorer resolution. The aim of this study was to identify as many arsenicals in the sample as possible, and thus the PRP-X100 was chosen.

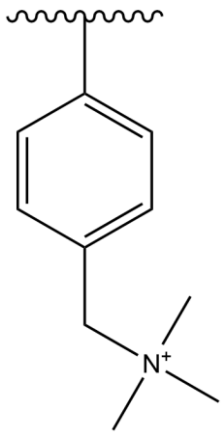


Figure 5-5: Chemical structure of the packing material of the Hamilton PRP-X100 column[127]. The trimethyl ammonium exchanger interacts with anions in the mobile phase, allowing resolution of neutral and anionic species in sample.

While the PRP-X100 allows effective separation of iAs^{III}, DMA, MMA and iAs^{V}, it is often difficult to separate the cationic and neutral species that are not retained from iAs^{III} which is uncharged at neutral pH. Since AsB, typically at high levels in oysters, can co-elute with uncharged iAs^{III}, it is common to use mobile phase of a higher pH to allow separation of iAs^{III} from AsB. LC methods in the literature included both isocratic and gradient methods, mostly using either NH_4HCO_3, $NH_4H_2PO_4$ or a combination of both to prepare pH adjusted buffer solutions as the mobile phase[125].

An isocratic method with reference to a study by Shimoda et al. [128] was employed for the initial trial run after considering parameters described in multiple reviews[28, 125, 129]. The reason for choosing an isocratic method is to minimize baseline drifts due to the difference in purity of eluents in binary mobile phase systems. This initial method using 30 mM NH_4HCO_3 adjusted to pH 8.5 as the mobile phase was tested by a mixture of 4 aresnical standards including AsB, iAs^{III}, DMA, and iAs^{V}. Resolution of the 4 standards was satisfactory, and great linearity was achieved for the calibration curves of individual arsenicals (Figure 5-6). The resolution between the AsB and the iAs^{III} can be improved, but the overall performance was adequate.

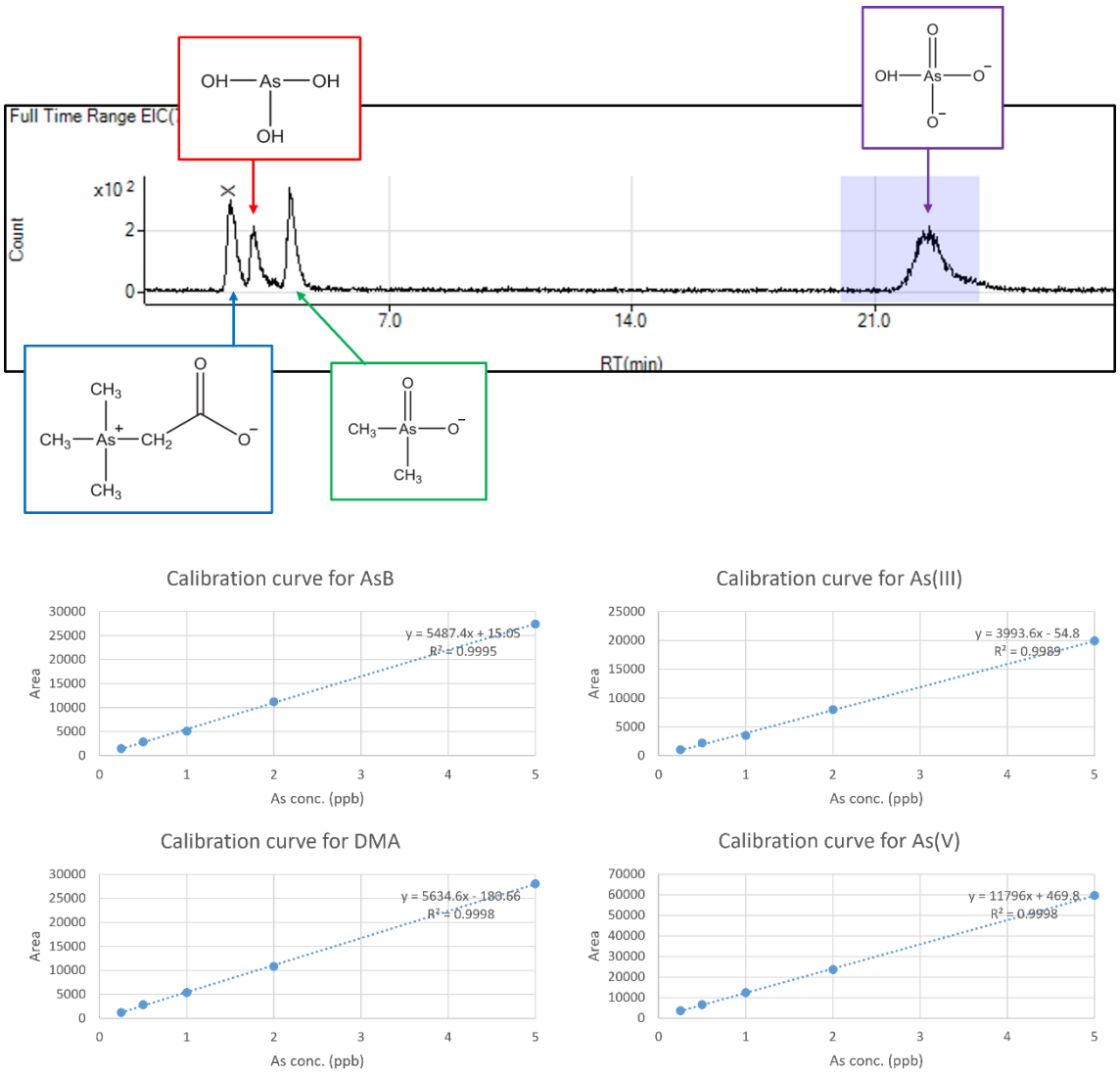


Figure 5-6: LC-ICP-MS chromatogram of 0.25 ppb standard mix and calibration curves of individual arsenical standards using the isocratic trial method.

Repeatability of the isocratic trial method, however, proved to be problematic. Complete resolution of the AsB and the iAs^{III} peaks was not consistently achieved (Figure 5-7). It was also found that carryover peaks were sometimes observed in blank injections (Figure 5-8), particularly if the blank injection was the first of the day after leaving the LC-ICP-MS idle overnight. Looking at the retention time of the carryover peaks, it is possible that

carryover peaks can overlap with the AsB, iAs^{III} and DMA peaks, causing broadening or shouldering of peaks and ultimately interfering with analysis.

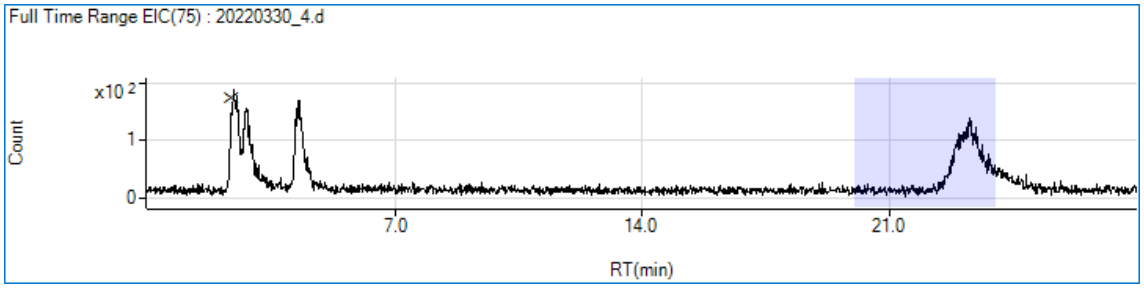


Figure 5-7: Chromatogram showing poor resolution of AsB and iAs^{III} peaks.

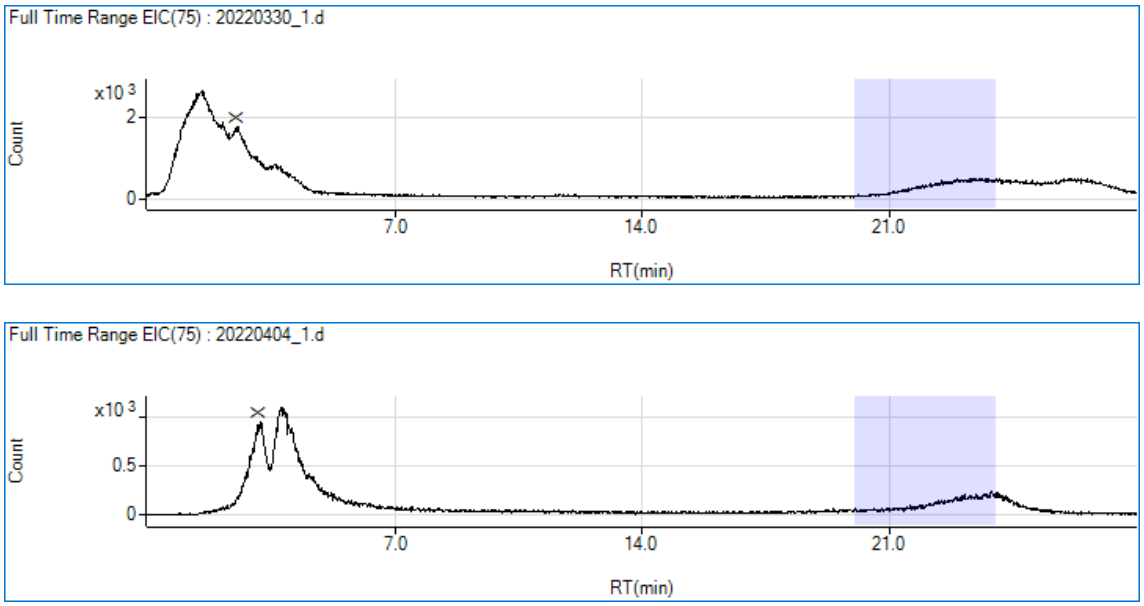


Figure 5-8: Carryover peaks seen in the first blank injection after idling overnight. Note that the carryover peaks are in some cases well defined and matched the retention time of the peaks of arsenical standards.

Other adjustments besides mobile phase selection were made after the trial runs revealed issues with the current method. One adjustment was that the integration time for the ICP-MS detection was increased from 0.2 second to 1 second to cope with low sensitivity and

noisy baseline. Increasing the integration time greatly reduced the noise to give a smoother chromatogram, as well as improved sensitivity at the expense of a slight reduction of resolving power. Another adjustment was that the protocol was changed to always analysing samples within the 24 hours of extraction. It was found that arsenical levels can change after extraction, especially with iAs^{V}. Change in iAsV level was observed even in standards (Figure 5-9), but it was unclear whether the change in level was due to transformation. As precaution, all standards and samples were prepared or extracted no more than 24 hours prior to instrumental analysis.

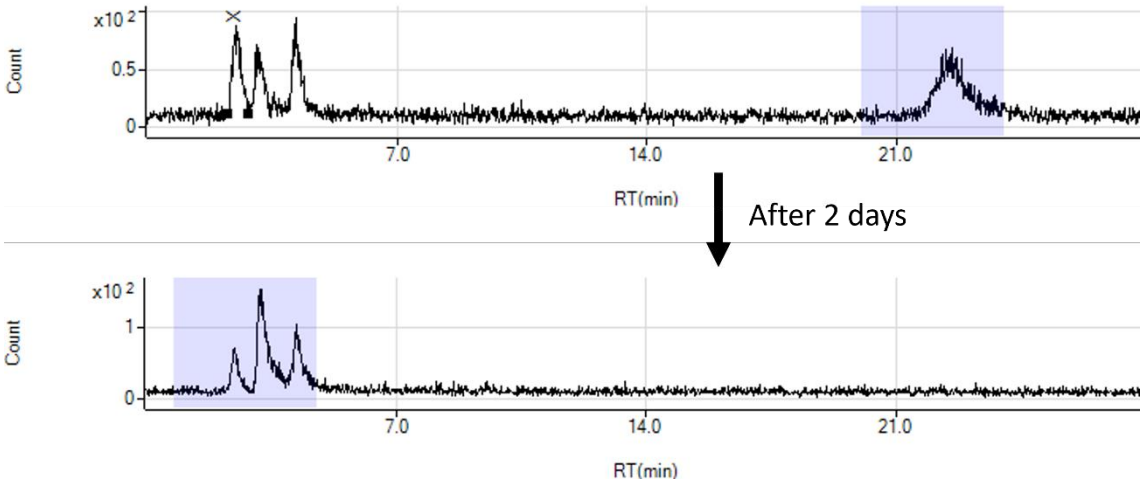


Figure 5-9: Chromatograms of the same standard mix when it was freshly prepared (top) versus 2 days after it was prepared (bottom). The iAsV peak (~ 22 minutes) was not observed in the chromatogram of the 2-day old standard mix.

To cope with the carryover issue, a gradient method using ultrapure water and 60 mM NH_4HCO_3 was used. The increase in concentration of NH_4HCO_3 was to ensure complete elution of arsenicals, especially the iAs^V ions that have strong interactions with the stationary phase. The chromatogram of the standard mix obtained using this method (Figure 5-10) exhibited a baseline drift, most likely due to the impurities in the NH_4HCO_3 salt used to prepare the mobile phase. An interesting point to note was that the use of a lower concentration of NH_4HCO_3 at the beginning of the run (10% 60 mM, therefore equalling 6 mM) did not lead to improved resolution of the AsB, iAs^{III} and DMA peaks.

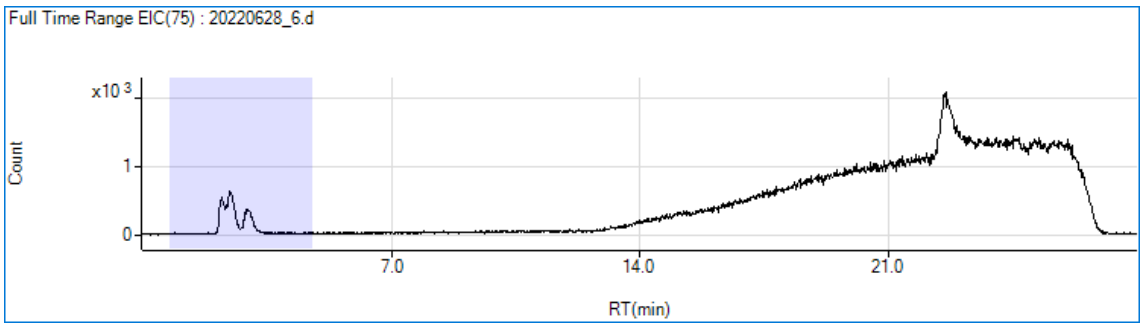
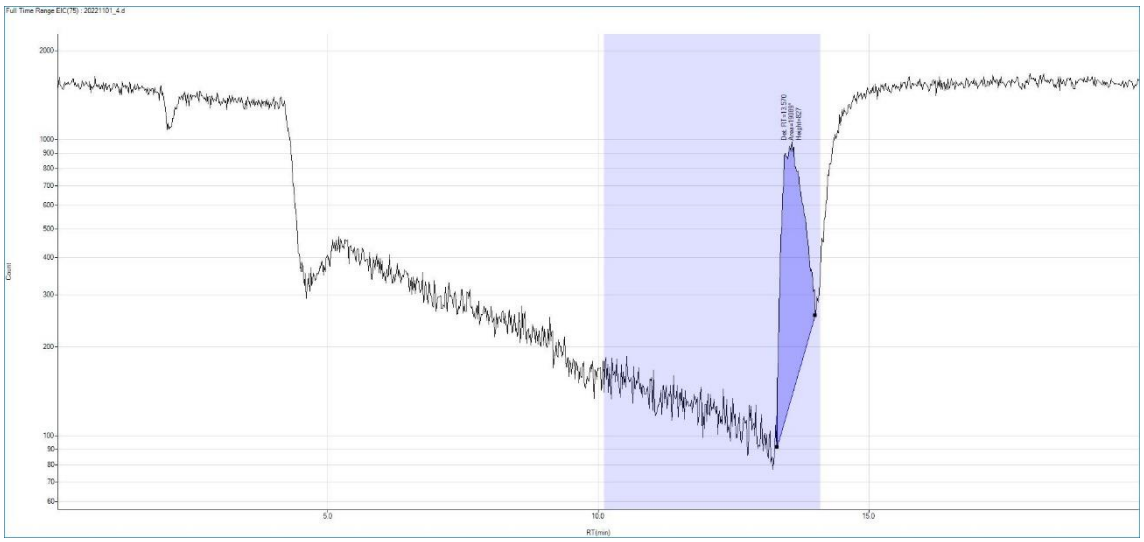


Figure 5-10: Chromatogram of standard mix using the first gradient method showing baseline drift and poor resolution of AsB, iAs^{III} and DMA peaks.

In order to effectively elute all iAs^{V} ions, especially the impurity from the mobile phase, the use of another salt as mobile phase was considered. Since the solute ion iAs^{V} is divalent, the use of divalent eluent ions should assist greatly in the effective elution of iAs^{V} ions[130]. The phosphate ion, a divalent anion and commonly considered a competing adsorbate to the iAs^{V} ion[131], was selected for this reason. A gradient program using 60 mM NH_4HCO_3 and 60 mM $NH_4H_2PO_4$ was developed, where $NH_4H_2PO_4$ is introduced after the elution of the DMA peak to facilitate quicker and more effective elution of iAs^{V}. The use of this gradient method resulted in the quicker elution of iAs^{V} at around 14 minutes compared to around 22.5 minutes in the previous methods. This allowed the shortening of run time down to 20 minutes, improving the analytical throughput significantly. Baseline drift was again observed due to the impurity in the mobile phase, and a peak is always observed at around 13.5 minutes, immediately before the elution of the iAs^{V} peak (Figure 5-11). This is likely due to the inadequate purity of the mobile phase reagent, leading to unwanted peaks, sometimes referred to as ghost peaks, in the chromatograms[132]. The NH_4HCO_3, being only of BioUltra grade, may contain traces of iAs^{V} that was retained in the column. As $NH_4H_2PO_4$ mobile phase was introduced, the iAs^{V} from the mobile phase is flushed out. Therefore, the peak caused by the impurity in the NH_4HCO_3 reagent is always eluted slightly before the peak of iAs^{V} in sample. Thus, while quantification of iAs^{V} may be affected due to the ghost peak, the qualification of iAs^{V} in samples was still possible.

Besides the ghost peak, the sensitivity of the method was also greatly affected by the impurity due to the baseline drift masking peaks of trace species. First, there was a sharp drop with the baseline at around 4 minutes, which can interfere with the integration of the DMA peak slightly. Second, there was a downward baseline drift from minute 5 to 13 with a lot of fluctuations, possibly masking any arsenical peaks eluted between the DMA peak and the iAs^{V} peak. However, despite the shortcomings with this method, it was successful in ensuring minimal carryover, demonstrating the use of phosphate ions is beneficiary.



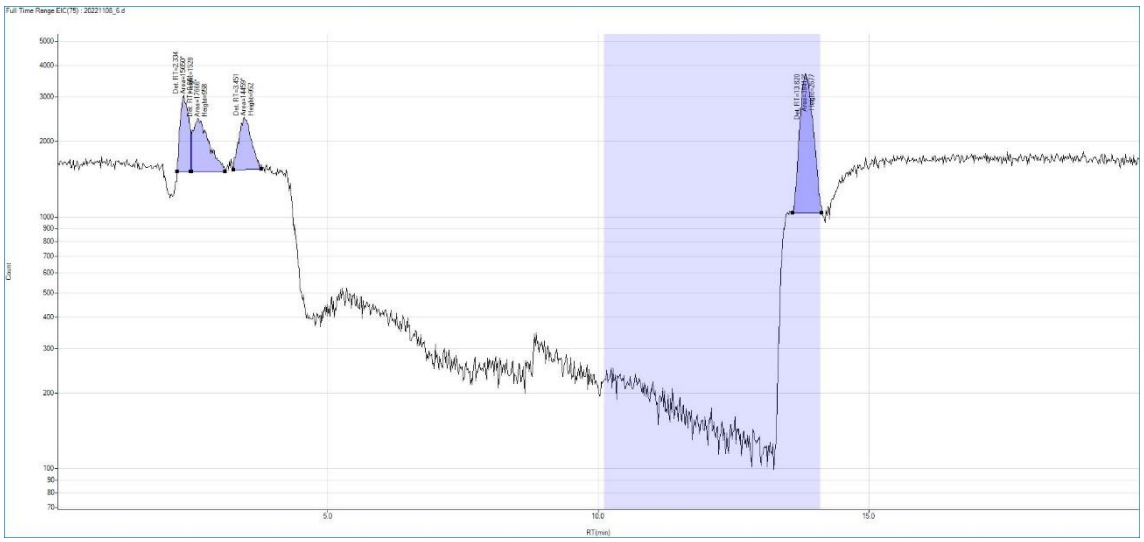


Figure 5-11: Chromatograms of blank (top) and 2 ppb standard mix (bottom) injections using the $NH_4HCO_3/NH_4H_2PO_4$ gradient method. The dip seen at 2 minutes is due to the injection containing ultrapure water and therefore less impurities than the mobile phase. The iAs^V peak in the 2 ppb standard mix chromatogram was not masked by the impurity peak, but the integration was affected.

Since the baseline drifts in the binary solvent gradient method is primarily due to the impurities with the BioUltra grade NH_4HCO_3, the use of only the trace metal grade $NH_4H_2PO_4$ in a gradient program was tested. A gradient from 3mM $NH_4H_2PO_4$ to 60 mM $NH_4H_2PO_4$ was used and the resulting chromatograms were an improvement from the binary solvent method. The resolution of peaks was similar to the binary solvent method while the baseline drift was less significant and the ghost peak issue was resolved (Figure 5-12). The downside of using this $NH_4H_2PO_4$ only method peak shape of the iAs peak was not as sharp, and that the resolution of peaks was still suboptimal. Nonetheless, this method performed the best in the methods tested and linearities of calibration curves were acceptable.

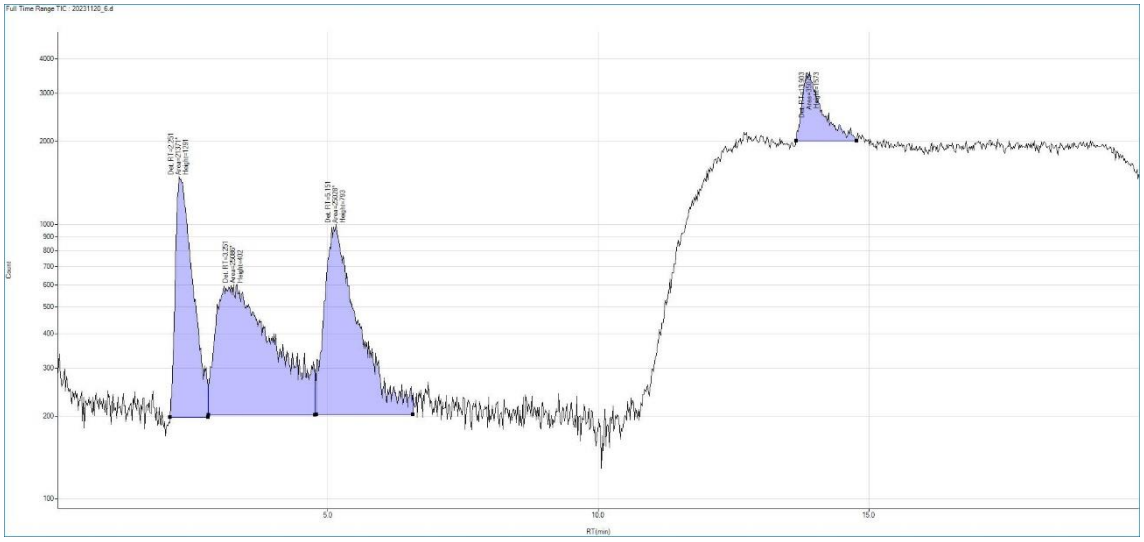


Figure 5-12: Chromatograms of 2 ppb standard mix injection using the $NH_4H_2PO_4$ gradient method.

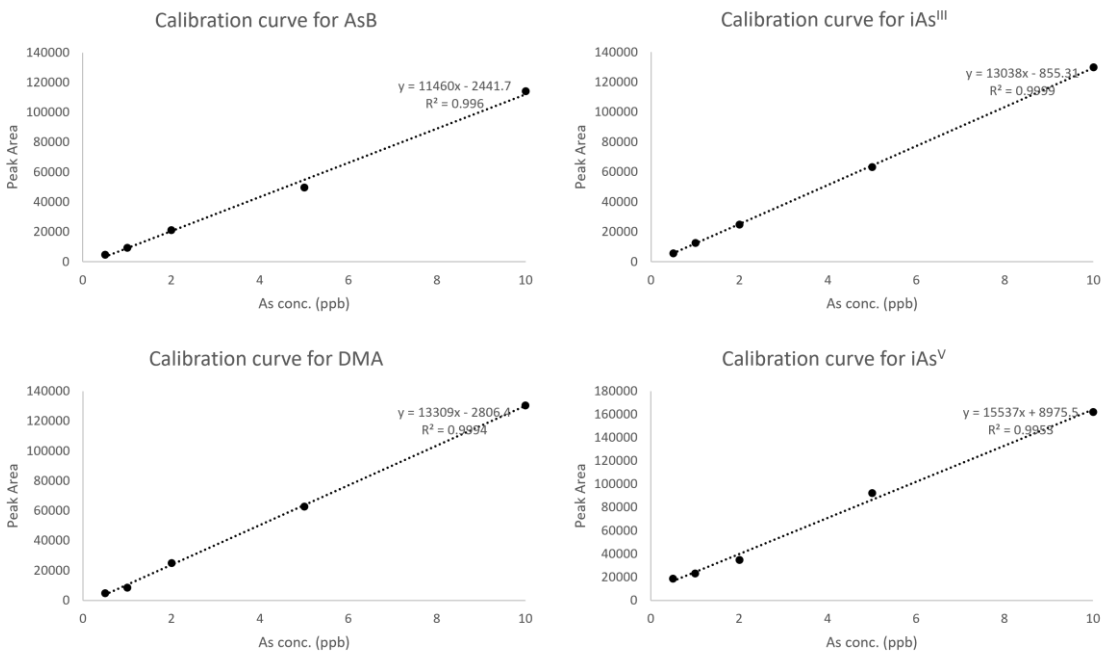


Figure 5-13: Calibration curves of individual arsenical standards using the binary solvent gradient method.

Thus, discussions on possible transformation of arsenicals were largely based on the results obtained using $NH_4H_2PO_4$ gradient method. However, accurate quantification of arsenicals was not attempted due to the difference in signal intensities of the different species as evident in the difference in the slopes of the fitted curves. Unknown peaks cannot be quantified because the relative signal intensities of these peaks were unavailable without corresponding standards. Thus, the discussion on possible transformation will be based on the relative amount of arsenicals in samples instead of absolute quantities.

Further studies on improving the resolution of peaks and minimizing carryover and baseline drifts are needed. The sole use of $NH_4H_2PO_4$ at low concentrations was employed for LC-ICP-MS but the limited volatility of phosphate ions can affect the signal

strength in ESI-QTOF, and therefore may not be optimal for the identification of arsenicals by matching retention times of peaks from chromatograms of ICP-MS and ESI-QTOF. The use of reagents of higher grade for the mobile phase should improve the ESI-QTOF signals.

5.3.2. Identification attempt using LC-ESI-QTOF

Identification of arsenicals was attempted using LC-ESI-QTOF. Since the aim of this part was solely for qualification instead of quantification, no calibration curve was prepared. AsB standard was injected using the positive ion mode to check for possible retention time shift due to the use of methanol and to see if the fragmentation pattern was in agreement with the literature. The MS1 and MS2 chromatogram and spectra are shown in Figure 5-13 and Figure 5-14 respectively.

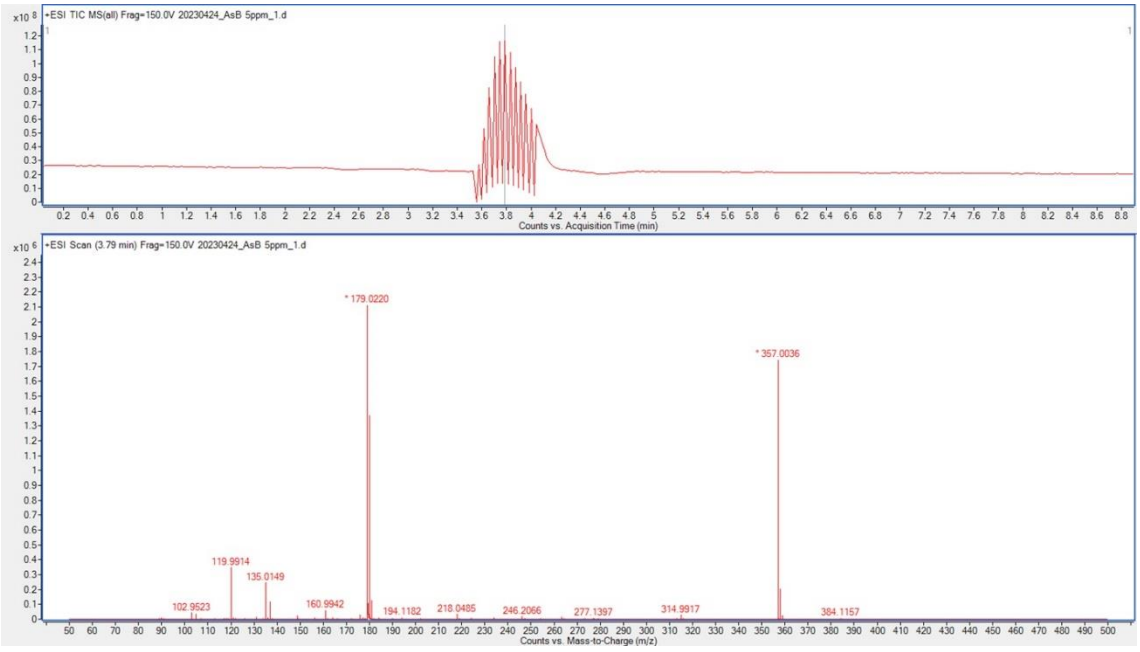


Figure 5-14: MS1 chromatogram (top) and spectrum (bottom) of an injection of AsB standard.

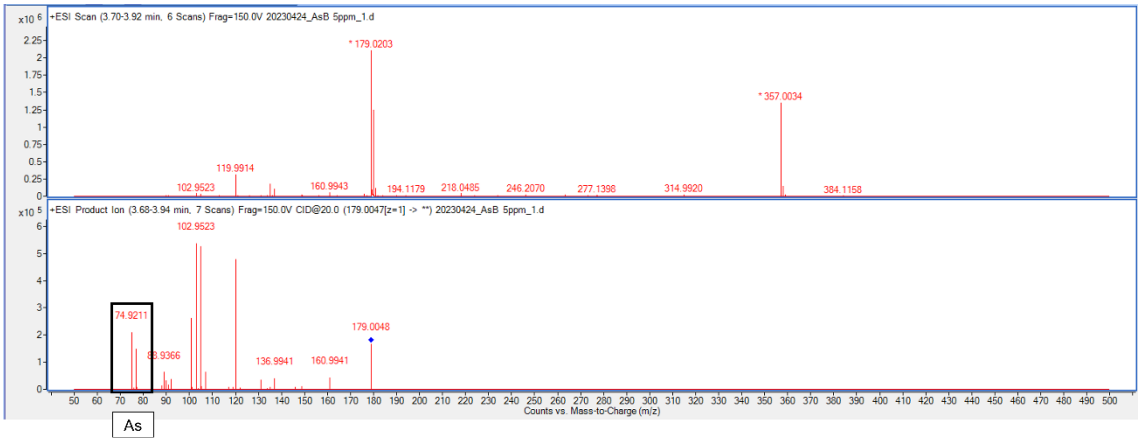


Figure 5-15: MS1 spectrum (top) and MS2 product ion scan spectrum (bottom) from the 179.02 *m/z* peak.

The tandem MS spectra of the AsB standard matched the reported values in the literature[118]. 179 *m/z* peaks, which corresponds to the intact AsB ion, were seen in the MS1 spectrum, the precursor ion spectrum, and the product ion spectrum. Fragments seen in the product ion spectrum matched the reported values in the literature: 161 *m/z* for the $(CH_3)_3AsCHCO^+$ fragment, 137 *m/z* for the $(CH_3)_3AsOH^+$ fragment, 103 *m/z* for the CH_3AsCH^+ fragment, and most importantly 74.92 *m/z* for the As^+ fragment. Detection of AsB was first tested because of the relatively high concentrations of AsB in the samples, and therefore making AsB likely to be the most easily detected arsenical in the sample.

Unfortunately, trial runs using samples yielded unsatisfactory results. Extract from the residues is used as an example here because it is of the highest level of arsenic in all of the samples, and identification of arsenicals, or at the very least AsB, should be less

challenging relative to the other samples. The MS1 and MS2 chromatogram and spectra are again shown in Figure 5-15 and Figure 5-16 respectively. It can be seen that in the MS1 spectra there is a peak with an *m/z* of 179.0049, which is only slightly off the *m/z* of the AsB ion and was very promising. However, a product ion scan using 179 *m/z* as the precursor did not show any of the previously mentioned fragmentation peaks specific to the AsB ion. Critically, the As^+ ion at 74.92 *m/z* was not observed in the product ions. A precursor ion scan was also conducted, but did not yield any peaks that matched literature *m/z* values.

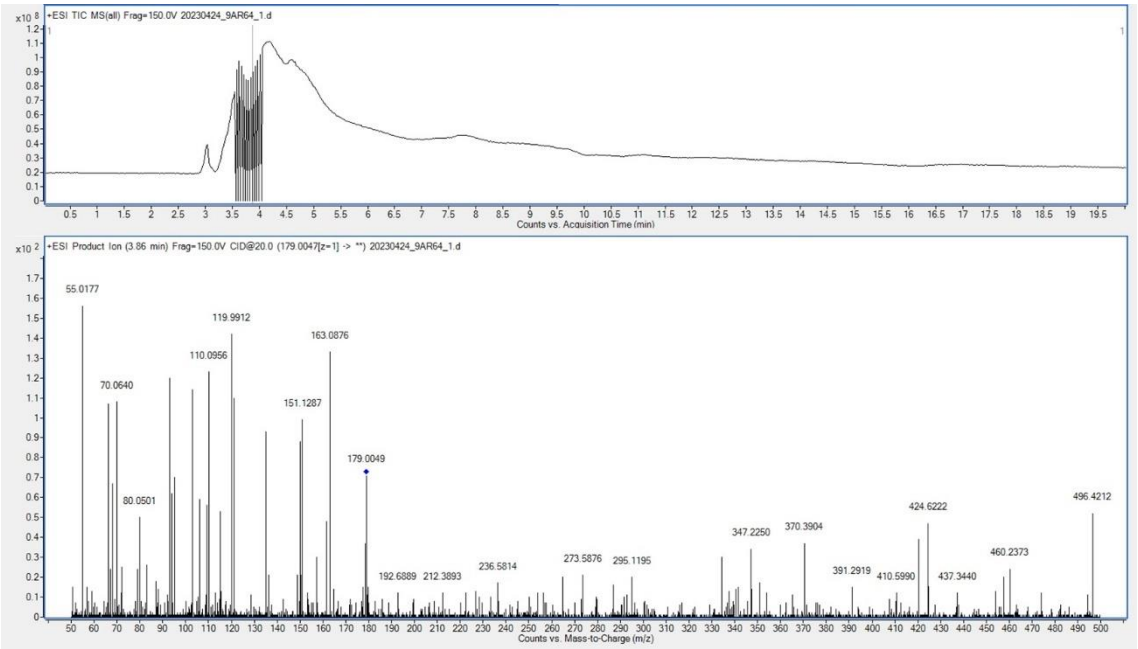


Figure 5-16: MS1 chromatogram (top) and spectrum (bottom) of an injection of the extract from deep-frying residues.

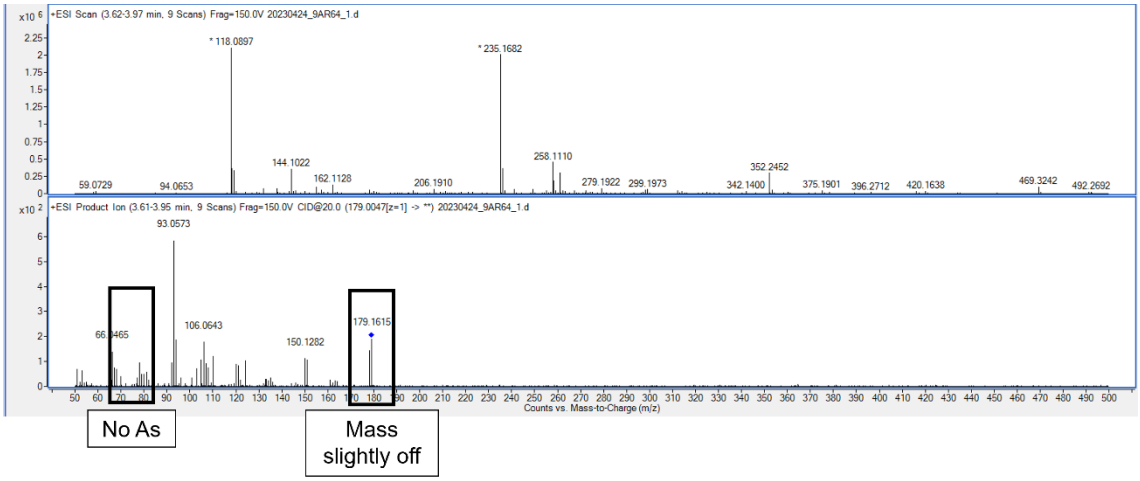


Figure 5-17: MS1 spectrum (top) and MS2 product ion scan spectrum (bottom) from the *m/z* 179 peak.

To conclude, the LC-ESI-QTOF analysis required further optimization before useful results can be yielded. A focus should be placed on the effective resolution of arsenical peaks in the LC before optimization of the ESI-QTOF, as better differentiation of arsenicals should enable easier detection and identification of individual species. As mentioned previously, the mobile phase of the LC can affect the ionization efficiency and therefore needs to be taken into account when developing a LC method.

5.3.3. Possible arsenic species transformation during deep frying

Change in arsenic species is critical for evaluating the change in risk of consumption as the toxicity of arsenic changes with the species. To identify if there are any changes in the species after deep-frying, the speciation profiles of raw oyster meat and deep-fried oyster meat were compared. The speciation profile of residues was also studied as the residues can be heated in the deep fryer for prolonged periods of time if not removed from

the deep-frying oil, possibly leading to drastic changes in the distribution of arsenic species.

As discussed previously, the signal strength of different species can vary and therefore absolute quantification cannot be achieved without standards for all the compounds. Thus, this study will focus on comparing speciation profiles to identify relative changes. Distribution of species in raw oysters was first studied. Five peaks were identified in the chromatogram of the raw oyster sample (Figure 5-17, comparison of samples with spiked samples in Figure S-1), including 3 peaks that did not match the retention time of available standards, and 2 peaks matching the retention times of AsB and DMA at 2.3 minutes and 5.1 minutes respectively. Relatively confident assignment can be made for the second peak as AsB. The second peak is the peak with the highest percentage peak area at 63±7%, which is consistent with the fact that AsB is expected to be the most abundant arsenic species in raw oysters[121, 133]. The assignment of the fourth eluted peak (percentage peak area 2.6±0.8%) as DMA is also relatively confident because the peak is well differentiated from adjacent peaks, and that the peak shape is well-defined. However, it is possible that these peaks, especially AsB, have overlapped with peaks of other trace species and masked them.

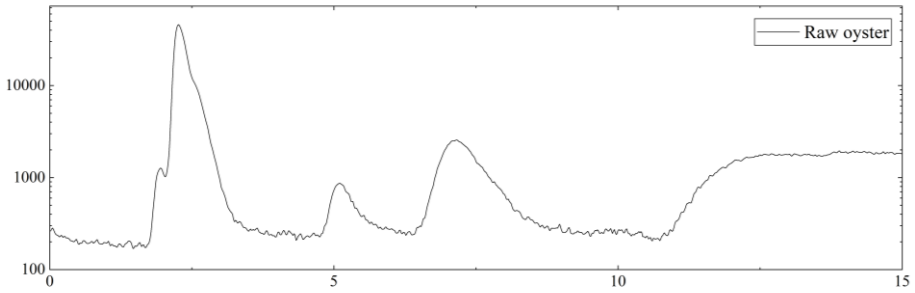


Figure 5-18: Chromatogram of arsenic speciation analysis of raw oyster sample. The peaks from left to right were (A) unknown 1, (B) AsB, (C) iAsIII, (D) DMA, and (E) unknown 2 by matching the retention times.

The peak that is eluted immediately after the AsB peak with a retention time of 2.5 minutes demonstrate some tailing and overlaps with the iAs^{III} peak but is believed to be of another species because iAs^{III} is at undetectable levels or only in trace amount in oysters according to literatures[121, 133-135]. This peak with a retention time of 2.5 minutes was possibly the TMAO peak. TMAO is commonly reported as one of the arsenic species found in oysters, and its retention time is reported to be between the AsB peak and the iAs^{III} peak[121]. The poor resolution of peaks together with the tailing of the large AsB peak led to difficulties in differentiating the TMAO peak from the AsB peak, and therefore quantification is challenging.

The identity of the other two unknown peaks can be speculated by comparing the elution sequence of the sample to the literature and the reported distribution of arsenic species of oysters in the literature. The first unknown peak is eluted immediately before AsB at 1.9 minutes, and its percentage peak area is at 1.2±0.1%. This strongly suggest that this first unknown peak is likely AsC, a species commonly found in oysters at lower levels and is eluted immediately before AsC when using PRP-X100 LC columns[121]. However, it should be noted that the peak might be a mixture of multiple cationic arsenicals minimally retained by the anion exchange column.

The remaining unknown, which is last eluted at 7.1 minutes, is of moderate size with a percentage peak area of 16±3%. In a study on oysters by Nam et al. [121], which also used

the PRP-X100 but used only NH_4HCO_3 as mobile phase, found a peak for an unknown compound that was eluted shortly after DMA and before MMA. It is possible that the unknown peak observed here has the same identity as the peak observed by Nam et al[121]. In summary, the peaks are assigned as AsC at 1.9 minutes, AsB at 2.3 minutes, TMAO at 2.5 minutes, DMA and 5.1 minutes, and the peak at 7.1 minutes unassigned.

Besides the peaks identified using the finalized LC method, interesting results were also obtained in the trial runs for the optimization of the LC method. A total of two unknown peaks were eluted before the elution of the AsB peak (Figure 5-18) when using the trial gradient method of increasing concentration of NH_4HCO_3. One of these two unknown peaks is again likely to be AsC as the compound is commonly eluted before AsB. It is reported in the literature[136] that the peak possibly consisted of coeluted cationic arsenicals due to the inability for the anion exchange column to retain cations. However, besides cationic arsenicals, Santos et al. [137] reported that iAs^{III} was eluted before AsB. In consideration of this, assignment is not made for the unknown peak due to a lack of confidence.

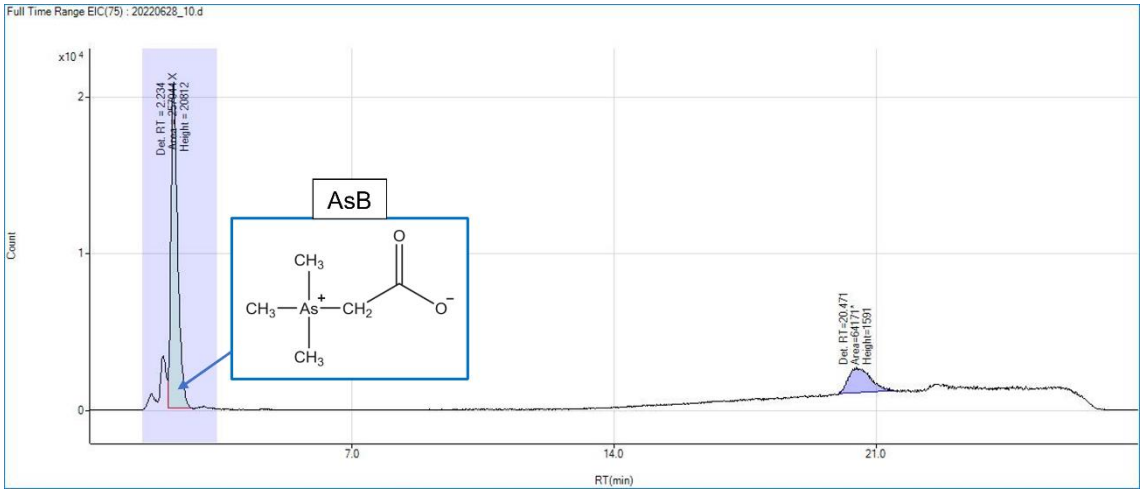


Figure 5-19: Chromatogram of trial arsenic speciation analysis of raw oyster sample. Two peaks are observed before the largest peak, which was assigned as AsB.

Speciation profiles of the deep-frying oil, the deep-fried oyster and the residues (Figure 5-19), all after 64 deep-frying cycles, were compared with each other as well as that of the raw oyster to check for any transformation or preferential migration of arsenicals. The chromatogram of the deep-frying oil contained 5 peaks, where 2 of them matched the retention times of AsB and DMA. The retention time matching was confirmed by performing a sample spike. It was interesting to see that the peak with the largest percentage peak area was, instead of AsB, the AsC peak at 48±2% in the deep-frying oil sample. The percentage peak area of the AsC peak was only at 1.2±0.1%. in raw oyster, much lower than in the deep-frying oil. The percentage peak area of DMA in the deep-frying oil was also significantly different to that of the raw oyster: 21±5% in the deep-frying oil versus 2.6±0.8%in the raw oyster, which is close to a ten-fold difference.

Similar phenomena were observed for the speciation profiles of the deep-fried oyster and the residues. The difference between the deep-fried oyster and the raw oyster was less significant, most likely because the core was protected from direct heat and interaction with the oil – only the surface of the oyster would interact with the hot oil. Nevertheless, percentage peak area of the AsC peak was higher at 6.9±0.3% compared to 1.2±0.1%, and the DMA peak at 4.4±0.3% compared to 2.6±0.8%. As for the residues, the percentage peak area of the AsC peak was at 65±1% and the DMA peak at 16±2%. Looking at the overview of the percentage peak area (Table 5-2), it was apparent that the relative levels of AsC and DMA were higher when deep-frying was involved, and was in the order of raw oyster < deep-fried oyster < deep-frying oil ≈ residues for both of the

peaks. This order was interestingly an ascending order of time of exposure to the hot oil: the temperature of raw oyster was never exposed to the oil, followed by the deep-fried oyster as the surface should be in contact with the oil for 3 minutes but the core would be protected from the oil. The residues would be in contact with the deep-frying oil for prolonged periods of time, and exposure of the core would be a minimal matter due to the fineness of the residues. It appears that the concentrations of AsC and DMA are proportional to the level of exposure.

Table 5-2: Percentage peak areas for raw oyster, deep-frying oil, deep-fried oyster, and residues (2 significant figures).

	AsC	AsB	TMAO	DMA	Unknown
Raw oyster	1.2±0.1%	63±7%	18±3%	2.6±0.8%	16±3%
Deep-fried oyster	6.9±0.3%	69±1%	13±1%	4.4±0.3%	6.3±0.4%
Residues	65±1%	15±2%	2.3±0.1%	16±2%	1.2±0.3%
Deep-frying oil	48±2%	12±3%	16±1%	21±5%	2.9±0.5%

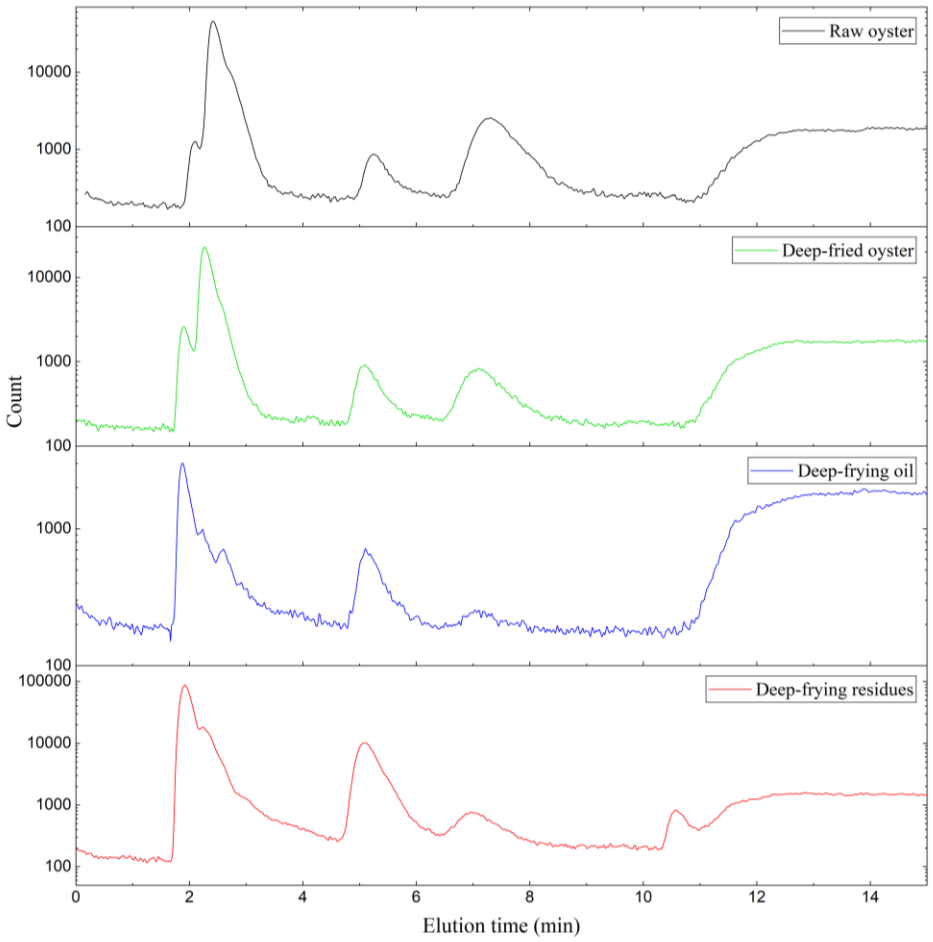


Figure 5-20: Arsenic speciation chromatograms for raw oyster, deep-fried oyster, deep-frying oil, and residues.

Conversely, the percentage peak area for some of the peaks decreased because of deep-frying. The unknown peak followed an order that was the exact reverse of that observed in the increased peaks: raw oyster > deep-fried oyster > residues ≈ deep-frying oil. This was a descending order of time of exposure to the deep-frying oil. The AsB peak also generally followed the mentioned order, where the percentage peak area of the raw oyster and deep-fried oyster are higher than that of the residues and the deep-frying oil. Integrating the two observations regarding the trends, the exposure to the oil most likely

played a role in the arsenic speciation profile of samples, which is consistent with literature describing the change in arsenic species under cooking conditions[138, 139].

For the TMAO peak, the relative levels were similar in all materials except for in residues, where the percentage peak area was significantly lower than the others at 2.3±0.1%. Further studies revealing the species contributing to the peak should provide more insight into the underlying cause.

One last yet critical observation was made in the chromatograms of the residue samples. In addition to the changes in relative amounts of existing arsenicals, a peak (0.59±0.31%) was observed at 10.6 minutes for the residue samples. This peak was not observed in any of the other samples tested. A comparison of the arsenicals elution order as well as the ratio of retention times with the literature[121] suggested that this peak most correspond to MMA. MMA, like DMA, is a carcinogen and an increase in its content could lead to increased food safety risks. The emergence of MMA in residues suggested the occurrence of transformation of arsenicals during the deep-frying process. The structure of MMA contains only one methyl group in the coordination complex of arsenic, which suggests that the high temperature in the deep-frying environment can lead to stripping of organic ligands from the complex. Since methylation of arsenicals is generally considered a detoxifying action, the demethylation caused by deep-frying would be an increase in toxicity of arsenicals involved in the deep-frying process. However, it is also possible that the difference between speciation profiles of the arsenicals is caused by preferential migration of arsenicals from the food to the deep-frying oil. For confirmation of the occurrence of arsenical transformation, quantitative analysis to quantify each of the

species in all components of the deep-frying process and a controlled experiment by spiking arsenical standards into batter can be attempted in future studies.

Speciation profiles of the raw grouper fish fillet and corresponding deep-fried fillet and deep-frying oil (Figure S3) were also studied. Speciation study of the residues from grouper fish was not conducted due to the insufficient amount of sample. There were changes in the relative peak intensities, as the relative levels of AsB decreased and relative levels of AsC increased as a result of deep-frying. The results were consistent with that of the oyster experiments, suggesting potential transformation of arsenicals with different food types instead of limited to with oysters.

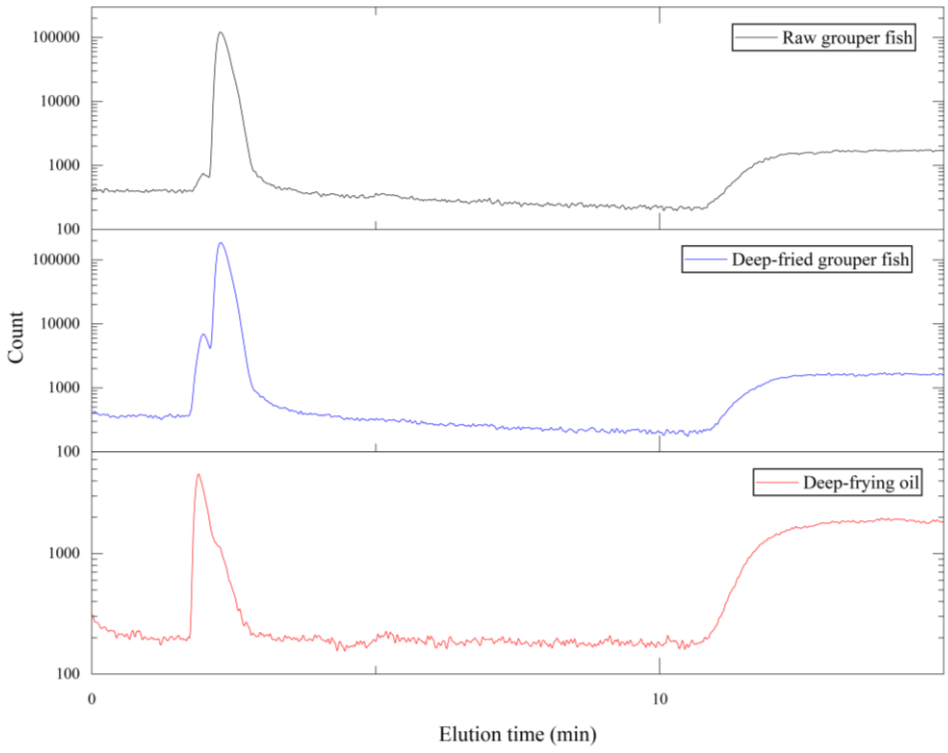


Figure 5-21: Arsenic speciation chromatograms for raw grouper fish fillet, deep-fried grouper fish fillet, and deep-frying oil.

On a concluding note, it should be noted that since accurate quantification of arsenicals was not attempted, the recoveries of each arsenic species as well as the overall recoveries were unavailable. Therefore, the changes in arsenic species profile can be affected by the preferential migration of arsenicals during the deep-frying process. However, transformation of arsenicals was proved possible as MMA, an arsenic species not observed in raw oysters, was found in the residue samples. In all, the most important finding from this study was that food safety risks can be heightened due to the increase in the relative levels of DMA and the transformation into MMA.

5.4. Conclusions

Speciation analysis of arsenic was attempted in this study with limited success. Several LC methods described in the literature were employed, but resolution of arsenical peaks was less than satisfactory. At the same time, attempts of using LC-ESI-QTOF to identify species were unsuccessful and did not provide additional information for species identification. Identification was limited to matching retention times of poorly resolved peaks of existing standards with unknown peaks, and therefore there was a lack of confidence with peak assignment.

Yet, the results were noteworthy as they suggested that deep-frying caused transformation of arsenicals. The speciation profile of the raw oyster was significantly different from the profiles of the deep-frying oil, deep-fried oyster or the residues. Comparisons between samples suggested the possibility of arsenic transformation into DMA, a weakly cytotoxic carcinogen[140]. It was found that the ratio of DMA compared to other arsenic species was higher in residues compared to the raw oysters. The change in relative amount of DMA can imply an increase in risks of consumption. Most critically, transformation of arsenic species into MMA was observed in the deep-frying residues. As toxicities of the transformed arsenicals are likely higher after demethylation, deep-frying causing demethylation indicates deep-frying food containing arsenic can lead to an increase in toxicity.

Further studies are needed to identify the arsenicals, both through better resolution of peaks and through optimization of LC-ESI-QTOF to allow studying fragmentation patterns of spectra. More concrete conclusions can be drawn once the identities and the

quantities of involved arsenicals are revealed; but the fact remains that food safety risk with foodstuff containing arsenic is likely increased during deep-frying.

Chapter 6:Overall conclusions and prospects for future research

Accumulation of heavy metals in deep-frying oil was investigated in this study using foods with high arsenic levels. It was found that arsenic accumulation was possible, reaching concentrations above 500 ppb when deep-frying oysters which is above the legislation limit for edible oil in Hong Kong. Arsenic content was particularly high in the residues formed during deep-frying, and that the high arsenic levels observed in deep-frying oil was mainly due to the presence of the residues. These residues also had a much higher lead concentration compared to the foodstuff, demonstrating heavy metal enrichment was not limited to arsenic and accumulation was possible but not observed in this experiment because of lower concentrations. Our investigation supported that these residues were formed from liquid leached from foodstuffs during the deep-frying.

Deep-frying experiments using grouper fish fillets yielded even more concerning results. The arsenic levels of the deep-frying oil and the residues were comparable to that of the oyster set of experiments, while the colours of both the oil and the residues were significantly lighter in the grouper fish fillet set. Deterioration of the deep-frying oil was also slower in the grouper fish fillet set, indicating that deep-frying oil contaminated with arsenic would less likely be replaced if grouper fish fillets were used. Residues being of a lighter colour was also problematic, as the food subsequently deep-fried would be of a lighter colour and therefore be more appealing to consumers. In short, consumption of food contaminated with arsenic is more likely and therefore the associated risk is greater.

Arsenic speciation analysis was attempted to evaluate the associated food safety risks. The preliminary results showed that the relative levels of arsenic species could change during deep-frying, with the relative levels of carcinogenic DMA and MMA increased. Changes in arsenic species distribution are correlated to the duration under heating, and

therefore the transformation of arsenicals might be more significant if the arsenic is not removed from the deep-frying oil in a timely manner, leading to an increase in the associated food risk.

In summary, significant accumulation of arsenic in repeatedly used deep-frying oil when frying foods such as oysters and grouper fish fillets was observed in this project. These results suggested the necessity of establishing guidelines and quality indicators for heavy metal contaminations in deep-frying oil. Removing residues from the frying oil and using batter to mask the food before the frying should be encouraged since they can significantly reduce accumulation of heavy metals in deep-frying oil, as demonstrated in our study.

Future studies are needed for a more thorough analysis of the potential risks associated with deep-frying foodstuff of high heavy metal content. The potential of accumulation of various heavy metals can be verified by identifying foodstuff contaminated with other heavy metals of concern, such as mercury, chromium and cadmium, and performing deep-frying experiments with the contaminated foodstuff. Other types of food such as vegetable products and fungi should also be tested as there are also reported cases of heavy metal accumulation in these types of food. Most critically, better speciation of arsenic is needed to qualify and quantify the arsenic species involved in the deep-frying process and in turn determine the associated food safety risks.

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Supplementary information

Table S-1: A consolidation of arsenicals studied in the literature from 2004-2018 by Ardini et al[28]. The large amount of occurring species adds to the difficulty of fully understanding metabolic pathways of arsenicals *in vivo*.

Full name	**Abbreviation**
Arsenite	iAs(III)
Arsenate	iAs(V)
Methylarsonate	MA
Dimethylarsinate	DMA
Arsenobetaine	AsB
Arsenocholine	AsC
Trimethylarsine oxide	TMAO
Tetramethylarsonium ion	TMA^+
Dimethylarsinoylethanol	DMAE
Trimethylarsoniopropionate	TMAP
Dimethylarsenoacetate	DMAA
Methylarsenite	MAs(III)
Dimethylarsenite	DMAs(III)
Phenylarsenate	PA
Dimethylarsenopropionate	DMAP
Oxo-arsenosugar-glycerol	Arsenosugar-Gly
Oxo-arsenosugar-phosphate	Arsenosugar-PO4

Oxo-arsenosugar-sulfonate	Arsenosugar-SO3
Oxo-arsenosugar-sulfate	Arsenosugar-SO4
Trimethyl-oxo-arsenosugar-glycerol	TMArsenosugar-Gly
Thio-arsenosugar-glycerol	Thio-arsenosugar-Gly
Thio-arsenosugar-phosphate	Thio-arsenosugar-PO4
Thio-arsenosugar-sulfonate	Thio-arsenosugar-SO3
Thio-arsenosugar-sulfate	Thio-arsenosugar-SO4
Arsenic-containing fatty acids	AsFA
Arsenic-containing hydrocarbons	AsHC
Arsenosugar-phospholipids	AsSugPL
Trimethylarsenio fatty alcohols	AsFOH
Arsenic – containing phosphatidylcholines	AsPC
Arsenic – containing phosphatidylethanolamines	AsPE
Thioarsenate	TAs(V)
Di-thioarsenate	DTAs(V)
Tri-thioarsenate	TriTAs(V)
Tetra-thioarsenate	TetraTAs(V)
Thio-methylarsonate/monomethylmonothioarsenate	Thio-MA
Dithio-methylarsonate/monomethildithioarsenate	Dithio-MA
Trithio-methylarsonate/monomethyltrithioarsenate	Trithio-MA
Thio-dimethylarsinate/dimethylmonothioarsenate	Thio-DMA
Dithio-dimethylarsinate/dimethyldithioarsenate	Dithio-DMA
Trimethylarsine sulftde	TMAS
Thio-dimethylarsinoylethanol	Thio-DMAE

b

Thio-dimethylarsenoacetate	Thio-DMAA
Arsenite phytochelatin complexes	As(III)-PC complexes

Table S-2: Microwave digestion program temperature setting. The final row is the completion and hence duration, maximum watt and target temperature are not applicable.

Time	Duration (minutes)	Maximum Watt (W)	Target temperature (°C)
00:00	0	1500	20
00:00	15	1500	100
15:00	3	1500	100
18:00	7	1500	150
25:00	3	1500	150
28:00	5	1500	180
33:00	25	1500	180
58:00	N/A	N/A	N/A

d

Table S-3: Typical operating conditions of ICP-MS. Some of the parameters listed here can be changed based on the results of tuning before the start of measurement.

Mode	He Mode
RF power	1550 W
RF Matching	1.20 V
Auxiliary gas	0.9 L/min
Plasma gas	15.0 L/min
He flow	4.0 mL/min
OctP bias	-18.0 V
OctP RF	200 V
Energy Discrimination	5.0 V
Nebulizer Pump	0.10 rps
Extract 1	-4.8 V
Extract 2	-245.0 V
Omega bias	-110 V
Omega lens	10.2V
Cell entrance	-40 V
Cell exit	-60 V
Deflect	0.0 V
Plate bias	-55 V
Mass gain	124
Mass offset	124
Axis gain	0.9994
Axis offset	0.06

e

QP bias	-13.0 V
Nebulizer	MicroMist (Agilent, part number: G3266-80004)
Sampler cone	Nickel
Skimmer cone	Nickel

f

Table S-4: Average percentage mass loss of foodstuff after deep-frying. The mass loss can indicate the loss of moisture content due to the deep-frying process.

	Oyster (no batter)	Oyster (battered)	Grouper fish fillet
% mass loss	52.7	20.7	36.4
SD of % mass loss	5.3	6.3	5.1

g

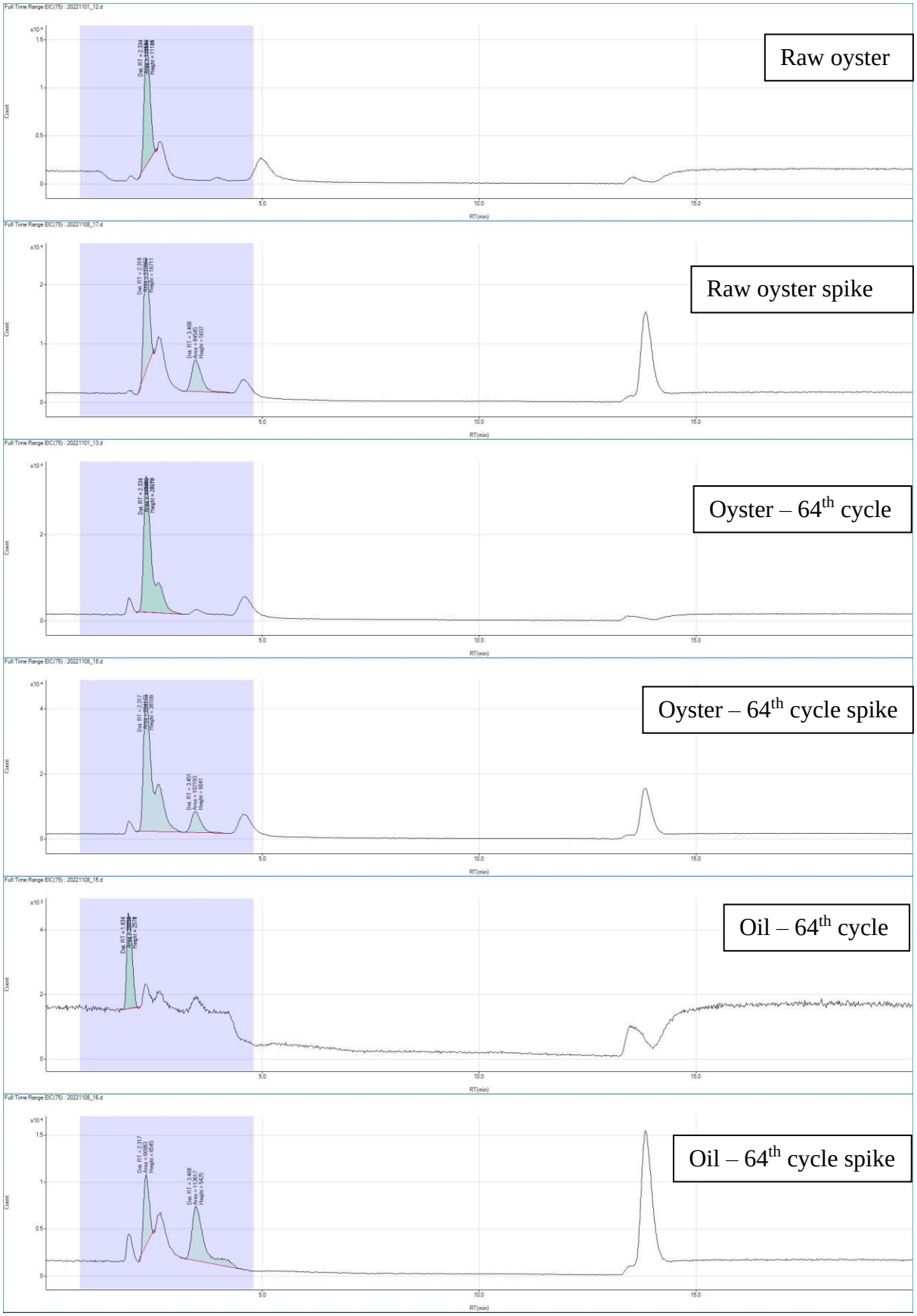

Figure S-1: Comparisons between chromatograms of samples and sample spikes.

h