Determination of Geosmin and 2-Methylisoborneol in Fish using *In-vivo* Solid Phase Microextraction

by

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Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The presence of off-flavor compounds in fish represents one of the significant economic problems encountered in aquaculture. These off-flavor compounds are due to the absorption of substances produced by microorganisms. Currently, a number of strategies have been employed to prevent or limit the growth of these microorganisms in recirculating aquaculture system. Therefore, it is important to evaluate the effectiveness of these strategies by monitoring the concentrations of off-flavor compounds in fish. *In-vivo* solid phase microextraction (SPME), a rapid and simple sample preparation method, allows the monitoring of concentrations of off-flavor compounds in live fish. In this research, geosmin and 2-methylisoborneol (2-MIB), which are produced by cyanobacteria and actinomycetes being the major sources for "earthy" and "muddy" flavors in fish, were selected as representatives. In order to accurately quantify these compounds in fish muscle, two kinetic calibration methods, on-fibre standardization and measurement using pre-determined extraction rate, were used. Results obtained were validated by traditional methods. The detection limit of *in-vivo* SPME in fish muscle was 0.12 ng/g for geosmin and 0.21 ng/g for 2-MIB, both below the human sensory threshold. Additionally, the binding effect of geosmin and 2-MIB in fish muscle was investigated in details. Facilitated by the agarose gel model, it was proven that binding did not impact the extraction rate under the pre-determined sampling time. Furthermore, an optional sampling position was undertaken by inserting the fibre into the fat tissue found under the fish belly, the results indicating that this method could decrease extraction time by up to two-thirds of its usual time.

Acknowledgement

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Dedication

I dedicate this thesis to my parents and friends for all their encouragement and support.

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Table of abbreviations

BCF ..... bioconcentration factor
CI ..... chemical ionization
CID ..... collision-induced dissocoation
CLSA ..... closed-loop stripping analysis
GC ..... gas chromatography
GC-FID ..... gas chromatography-flame ionization detector
GC-EI-IT-MS ..... GC-electron ionization--ion trap-mass spectrometry
GC-MS ..... gas chromatography-mass spectrometry
GSM ..... geosmin
LC ..... liquid chromatography
LOD ..... limit of detection
MLD ..... method detection limit
MD-SE ..... microwave distillation-solvent extraction
MD-SPE ..... microwave distillation-solid-phase extraction
MD-SPME ..... microwave distillation-solid-phas e microextraction
2-MIB 2-methylisoborneol
ND-SPME ..... negligible depletion-solid phase microextraction
PBS ..... phosphate-buffered saline
PDMS ..... polydimethylsiloxane
PEG ..... polyethyleneglycol
P&T-SE ..... purge and trap-solvent elution
RAS ..... recirculating aquaculture system
RSD ..... relative standard deviation
SIS ..... selected ion storage
S/N ..... signal-to-noise
SPME ..... solid phase microextraction

1. Chapter 1- Introduction

1.1 Off-flavor compounds in fish

1.1.1 Off-flavor compounds in fish raised in recirculating aquaculture systems

The fisheries sector is a major resource-sector industry in Canada related to water resources. Recently, aquaculture has been touted as a strategy to support the downfall of the fishing industry. The technology of fish farming in recirculating (or closed-circuit) aquaculture systems (RASs) is the next revolution in the field of aquaculture production.

RAS is systems in which water is (partially) re-used after undergoing treatment.[1] Environmental pressures, and in particular the growing interest to rationalize water use, are powerful incentives for adopting the use of environmentally friendly as well as flexible production techniques that will allow the industry to grow in a sustainable manner.[2] Currently, commercial RAS production systems typically recirculate over 99% of its water usage, significantly reducing water consumption.[3] In addition, RAS improves opportunities for waste management and nutrient recycling,[4] thus providing better hygiene and disease management,[5] as well as biological pollution control[6].

One major disadvantage in the current development of RAS is the presence of off-flavor compounds. These compounds found in farm-raised fish cause one of the most serious economic problems encountered in aquaculture related to product quality. The undesirable odors and/or tastes in fish are the cause of a major reduction in the consumption of such products, also rendering fish unfit for retail.[7] Among those flavors, the "earthy" and "muddy" odors constitute more than 80% of the off-flavor problems found in farm-raised fish.[8] Such flavors come from the absorption by fish of substances

including geosmin and 2-methylisoborneol, which are produced by a broad group of bacteria in water.[9-11]

1.1.2 Geosmin and 2-methylisoborneol (2-MIB)

Yurkowski and Tabachek first reported geosmin as the cause of the muddy flavor found in rainbow trout from natural sources.[12] Later, Persson and York found that 2-MIB is absorbed by rainbow trout in continuous-flow aquaria, producing a muddy flavor in its muscle.[13]

Geosmin and 2-MIB are tertiary alcohols, both existing as (+) and (-) enantiomers (Fig. 1.1).[14] Additionally, it has been reported that the natural form is the (-) enantiomers for both compounds.[15,16] In relation to the odor of geosmin, the (-) enantiomer has on average a threshold 11 times lower than the (+) one.[17] The odor of 2-MIB depends on the concentration, on the other hand. Persson *et al*. reported that pure 2-MIB exhibited a camphoraceous odor, while extremely diluted concentrations exhibited a musty or muddy odor.[18] Although the flavors released by these two compounds are found to be unpleasant, there are currently no regulations in place for their presence in fish produce, as they have not been associated with any health effects.

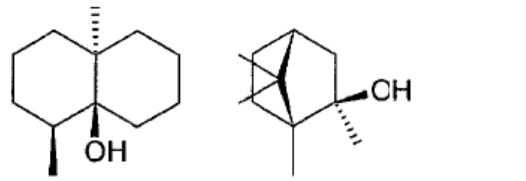


Figure 1.1 Structure of (-) geosmin and (-) 2-MIB[14]

It is very important to know the physical and chemical properties of a compound before analysis. Table1.1 shows the general characteristics/properties of the two analytes being studied. From the perspective of volatility, geosmin, whose boiling point is above 250 °C, belongs to semi-volatile compounds, while 2-MIB is classified as a volatile compound, indicating the possibility of analysis using gas chromatography (GC). Regarding to log K_{ow}, more details will be explained in the next section.

Table 1.1 Physical and chemical properties of geosmin and 2-MIB

	(-) Geosmin	(-) 2-MIB
Name[14]	[(-)-(4*S*,4a*S*,8a*R*)-4,8a-dimethyloctahydronaphthalen-4a-ol]	{(1*R*-exo)-1,2,7,7-tetramethylbicyclo[2.2.1]heptan-2-ol}
Molecular weight (g/mol)[19,20]	182.30	168.28
Boiling point (°C)[19,20]	270-271	208.7
log K_{ow}[19]	3.57	3.31

The occurrence of geosmin and 2-MIB was reported to be caused by cyanobacteria. Supporting this, enhanced odor concentrations had coincided with high densities of cyanobacteria[21-23]. On the other hand, the observations showed by Lanciotti *et al.* indicate that actinomycetes, possibly in association with microalgae, were the major odor producers during the winter in Arno River, Italy.[24, 25] Moreover, the presence and intensity of the taint are more prominent in eutrophic conditions, where overabundance of nutrients or warmer water presents.[26] Fig. 1.2 shows the formation pathway of geosmin and 2-MIB.

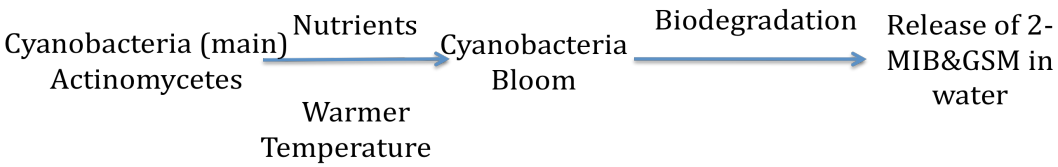


Figure 1.2 Formation pathway of geosmin (GSM) and 2-MIB[26]

1.1.3 Uptake of geosmin and 2-MIB in fish

When fish are exposed to tainted water containing geosmin and 2-MIB, they uptake these compounds and accumulate them in their tissue. Better understanding the process of uptake of geosmin and 2-MIB assists experimental design and results interpretation. The uptake of odor compounds by fish may be through several routes: gills, skin and alimentary canal.[27] Clark *et al*. reported that the uptake route for fish is related to the octanol/water partition coefficient of the chemical; uptake through the gills can dominate up to log K_{ow} of 6.[28] As mentioned previously, geosmin and 2-MIB with log K_{ow}'s of 3.57 and 3.31, respectively, uptake should happen overwhelmingly through the gills. This theory has been also proven by From *et al*. using rainbow trout.[29]

The concentrations of off-flavor compounds in the water and the exposure time are the two main factors affecting the amount of uptake.[30-32] In addition, species of fish, the physiological state of the fish, size of fish, water temperature, fat content of fish and environmental factors all might relate to uptake amount as reported.[21, 34-36]

When fish are exposed to tainted water, the compounds pass into the fish until the fluxes of the chemical into and out of fish are balanced and there is no net flow of compound through the gills. At this point, the concentration in the lipid phase is the concentration in the water phase of the fish times the lipid/water partition coefficient.

Therefore, the concentration of the chemical in the tissues of fish is greater than that in the water. This ratio is known as the bioconcentration factor (BCF).[21]

Fat content is a controversial factor in terms of uptake of geosmin and 2-MIB in fish. The thermodynamic model indicates that the concentrations of the chemicals found in the muscle of fish would vary among the fish and depend on the lipid content of tissue, although all the fish were exposed to the same ambient water.[21] For instance, Johnsen and Lloyd reported that fatter fish (>2.5% muscle fat) have a higher uptake rate and accumulation amount than leaner ones (<2%) for 2-MIB. However, later in another publication, they discussed that water temperature was the main factor instead of the fat content.[34] Simliar results were confirmed by other researchers.[21, 35] Additionally, when two different species of fish, catfish and rainbow trout were compared, no significant difference of the uptake rate and amount was observed.[36]

1.2 Solid phase microextraction *in-vivo* sampling

1.2.1 *In-vivo* sampling

Currently in RAS research, there is focus on developing strategies to prevent or limit the development of microorganisms that produce the off-flavor substances found in fish. Therefore, in order to evaluate the effectiveness of these stratergies on microorganisms, a method needs to be implemented that can monitor the level of target compounds by repeatedly sampling the individual living fish in RAS at different time points. In addition, during metabolism or toxicology studies, an *in-vitro* method may not accurately predict the fate of a xenobiotic, thus necessitating verification using an *in-vivo* model.[37] As well, error and elapsed time can be reduced with the elimination of sample

transport and storage.[38] Furthermore, from the perspective of animal ethics, *in-vitro* methods can cause severe damage to living organisms, or worse, demand their sacrifices. On the other hand, the *in-vivo* approach is a relatively non-invasive approach that minimizes experimental animal use. Currently, applicable *in-vivo* analysis techniques include microdialysis, sensors, microfluidics, nanomaterials, and solid phase microextraction.[37]

1.2.2 Comparison of *in-vivo* solid phase microextraction to other methodologies

An ideal *in-vivo* sampling technique should be miniature, solvent-free, as well as able to offer integration of sampling, sample preparation, and sample analysis steps.[37, 39] The invention of solid phase microextraction (SPME) in 1990 brought significant advantages, addressing all three challenges.[40]

SPME is a rapid, inexpensive and solvent free sample preparation method, which combines sampling, analyte isolation and enrichment into one step. Moreover, *in-vivo* sampling with SPME has its unique advantages due to its convenient device design. The needle-like device can be exposed directly into the living system, and after a short pre-determined extraction time, the device can be introduced into GC for thermal desorption, or desorbed using solvents before injection into liquid chromatography (LC).

Although microdialysis is the standard, when compared with *in-vivo* SPME, it shows significant drawbacks, such as loss of perfusion fluid, the need for a pump, poor performance for hydrophobic species, as well as a complicated calibration method.[37, 41, 42]

Several *ex-vivo* methods have been reported for determination of geosmin and 2-MIB in fish, including closed-loop stripping analysis (CLSA),[43] purge and trap-solvent

elution (P&T-SE),[34] microwave distillation-solvent extraction (MD-SE),[44] microwave distillation solid phase extraction (MD-SPE),[45] microwave distillation solid phase microextraction (MD-SPME),[46] and dynamic headspace sampling[47]. Compared to *in-vivo* SPME, they all need additional experimental equipment setup as well as specific amounts of fish muscle from sampling. CLSA and P&T-SE require minimum of 2 h and 3 h extraction time for an individual sample, respectively. MD-SE, MD-SPE are subjected to large amount of solvent use. The process of performing dynamic headspace sampling is too complicated and time consuming.

Each method is pursuing the lowest detection limit (Table 1.2), which should be at least lower than the human sensory threshold. Human sensory detection thresholds of geosmin and 2-MIB in fish are significantly dependent on the lipid component in the flesh: the greater the proportion of lipid the higher the value of the threshold. That is, higher amounts of the chemical need to be present in the flesh in order to reach the human sensory threshold.[21] Therefore, reported measurements of sensory thresholds of geosmin and 2-MIB in fish should be accompanied by a statement of fat content found in the material. Generally, farm-raised fish contain greater fat than wild fish.

Several studies have reported values of human sensory thresholds of geosmin and 2-MIB in rainbow trout (*Oncorhynchus mykiss)*. Yurkowski *et al*. reported the geosmin sensory threshold value in rainbow trout as 0.6 µg/100g,[12] which is similar to Persson's study (6.5 µg/kg).[48] Lovell *et al*. reported a value of 8.5 µg/kg for the threshold of geosmin in chanel catfish, which is the similar material to rainbow trout.[49] In another study, Robertson *et al.* found a threshold of 0.9 µg/kg for geosmin and 2-MIB in rainbow

trout, a figure almost eight times smaller than previous studies.[50] Persson and Lelana reported the sensory thresholds of 2-MIB in trout as 0.55 and 0.7 μg/kg, respectively.[51-52]

Table 1.2 Detection limit of published methods

Methods	Geosmin (μg/kg)	2-MIB (μg/kg)
P&T-SE/GC	-	0.05
MD-SE/GC	-	-
MD-SPE/GC-MS	0.630±0.109	0.217±0.018
MD-SPME/GC-MS	0.01	0.01
Dynamic headspace sampling/GC-MS	<0.1	<0.1

"-" means no information could be refered.

1.2.3 Fundamentals of *in-vivo* SPME

1.2.3.1 Kinetics of SPME

Direct extraction mode is the most widely used SPME sampling technique. It includes two steps to perform this extraction: first, the fibre (extraction phase) is exposed to the sample, and analytes with a high affinity are selectively extracted. In the second step, compounds extracted by the fibre are desorbed into the analytical instrument, allowing the fibre to be used repeatedly. Sometimes, a clean-up step needs to be added after extraction from very complicated matrices such as food samples.

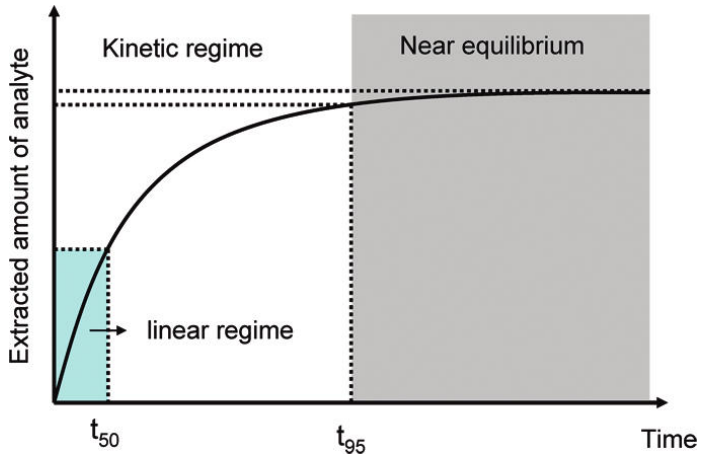


Figure 1.3 Typical extraction time profile of SPME.[53]

The extraction process of SPME generally follows the profile shown in Fig. 1.3. It can be seen from the graph that when the fibre is inserted into the sample there is an almost linear mass uptake process when the extraction time is less than t_{50} (50 % of equilibrium amount is extracted); afterwards, the rate of extraction slows down and eventually reaches equibilirum. Since the equilibrium time is infinitely long, t_{95} is often assumed to be the equilibrium time.[54]

At equilibrium time, the extraction amount by a liquid coating SPME fibre can be described by eq. 1.1, according to the law of mass conservation and thermodynamics of partition equlibrium; if only the sample matrices and the coating are considered:[54]

$$n_e = \frac{K_{fs} V_s V_f}{K_{fs} V_f + V_s} C_0 \qquad (1.1)$$

where n_e is the extracted amount at equilibrium; K_{fs} is the distribution coefficient of the analyte between the fibre coating and sample matrix, which is dependent on temperature, pH and the matrices composition; V_f is the coating volume; V_s is sample volume; C_0 is the

initial concentration of analyte in the sample. When V_s is much larger than $K_{fs}V_f$ (fibre constant), the eq. 1.1 can be simplified as eq. 1.2. For fish samples, since volume of fish is far greater than the fibre constant, eq. 1.2 can be used for quantification of target compounds in fish.

$$n_e = K_{fs}V_fC_0 \tag{1.2}$$

While eq. 1.1 can only express equilibrium SPME, the entire absorption kinetics of the analyte from the sample matrices to SPME liquid coating can be described by eq. 1.3[55]:

$$n = [1 - \exp(-at)]n_e = [1 - \exp(-at)]\frac{K_{fs}V_fV_s}{K_{fs} + V_s}C_0 \tag{1.3}$$

where n is the amount of extracted analyte at time t, n_e is the amount of analyte extracted at equilibrium, and a is a rate constant that is dependent on the volumes of extraction phase and sample, the mass transfer coefficients, the distribution coefficients and the surface area of the extraction phase.[54] When extraction time is long enough to reach equilibrium, eq. 1.3 is simplified as eq. 1.1. According to eq. 1.3, for pre-equilibrium extraction, there is a linear relationship between the fibre extracted amount (n) and the concentration of analyte in the sample matrix (C_0); if the convection conditions, the extraction time and temperature remain constant.[54]

1.2.3.2 Kinetic calibration methods

Successful use of *in-vivo* SPME is dependent on the selection of calibration method. Among existing calibation methods of SPME, equilibrium extraction, external

standard calibration, and kinetic calibration are the most suitable methods for *in-vivo* SPME.

Equilibrium extraction is an attractive option for rapid analysis with known fibre coating and sample matrix distribution coefficients of the analytes. For solid matrices such as fish, the diffusion route of an analyte in the solid tissue is longer than in a free solution due to the tortuosity.[56] Therefore, it is time-consuming and unethical to keep fibres inside fish for more than 10 hours, which is the equilibrium time for both analytes by using equilibrium extraction of SPME. An external calibration method requires availability of standard samples with similar matrices compositions, which is easier to perform in gas or liquid samples. However, for complex samples such as fish, it is difficult to accomplish.

Kinetic calibration methods, when compared with the two traditional calibration methods mentioned above, are newly developed and particularly useful for complex matrix *in-vivo* quantification. The theory of kinetic calibration was developed by Chen *et al*. based on Ai's proposed model shown in eq. 1.3.[57] In Chen's study, they demonstrated an isotropic behaviour between absorption and desorption in the SPME liquid coating, and named this calibration method as on-fibre standarization method.

Generally, the desorption kinetics of the standard from SPME fibre to the sample matrices can be represented by eq. 1.4:

$$Q = q_0 \exp(-at) \tag{1.4}$$

where Q is the amount of standard remaining in the extraction phase after sampling time t; q_0 is the amount of pre-loaded standard in the extraction phase; the constant a has the same definition as in eq. 1.3, and where analytes have similar physicochemical properties

such as isotropically labeled standards, the constant *a* should be the same for both desorption and absorption.[57] By combining eq. 1.3 and 1.4, the kinetic process can be expressed as eq. 1.5:

$$\frac{n}{n_e} + \frac{Q}{q_0} = 1 \tag{1.5}$$

After substituting n_e in eq. 1.2 with eq. 1.5, the intitial concentration of target analytes in the sample matrices, C_0, can be calculated by eq. 1.6:

$$C_0 = \frac{q_0 n}{K_{fs} V_f (q_0 - Q)} \tag{1.6}$$

Fig. 1.4 shows the absorption and desorption processes of the liquid coating of SPME fibre within the boundary layer of fish tissue.

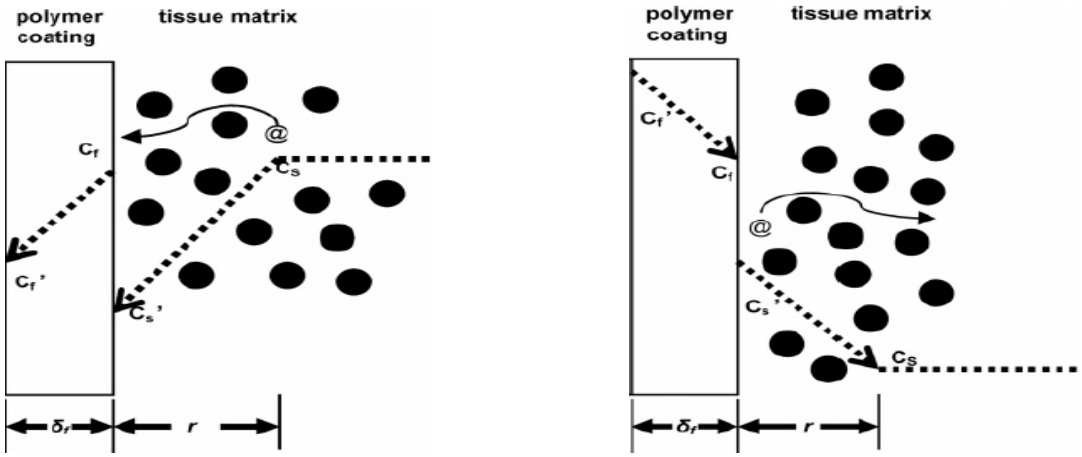


Figure 1.4 Schematic of the absorption and desorption processes between the liquid coating of SPME fibre and the boundary layer of tissue matrix. A linear concentration gradient is assumed in both the fibre coating and the tissue medium when the experimental conditions are constant. The left one is the absorption process and the right one is the desorption process. C_s is concentration of the analyte in the tissue matrix, C_s' is the concentration of the analyte in the sample at the interface of the fibre coating and the tissue, C_f is the concentration of the analyte in the coating at the interface of the fibre coating and the tissue, C_f' is the concentration of the analyte in the coating at the interface of the fibre coating and the fused silica core, and δ_f is the thickness of the fibre coating, r is the thickness of the boundary layer, @ represents an analyte molecule with the arrow line indicating the diffusion route, and the black dots are the solid tissue, which the analyte does not pass through.[56]

Based on the on-fibre standardization calibration method, some other kinetic calibration methods have been developed, including dominant pre-equilibrium desorption, one-calibrant kinetic calibration, as well as quantification using the pre-determined sampling rates of analytes. For dominant pre-equilibrium desorption, this calibration method may be too costly because different fibres are needed for both extraction and desorption. In addition, the extraction and desorption steps need to be performed separately and far from each other in case of pollution.[56] The one-calibrant kinetic calibration technique requires the knowledge of the diffusion coefficients of the target analytes and the calibrant in the sample matrix. Therefore, it is feasible for air or water sampling, since molecular diffusion coefficients in air or water can be easily obtained in literature or calculated with empirical equations, but it is not practical for direct sampling of analytes in blood or animal tissues.[37, 58] While the on-fibre standardization method has its own disadvantage when the isotropic standards are not available. However, in our situation, the deturated geosmin and 2-MIB were accessible. Thus, on-fibre standardization calibration is the first choice of quantification method.

Recently, another calibration method using pre-determined sampling rates of the analytes has been reported by Ouyang *et al*.[37] In this technique, it assumes that the rate of mass transfer or sampling rate remains constant throughout the duration of sampling within the linear range. The relationship between the concentration of target analytes in the sample matrices (C_0) and the extracted amount of analytes at time t (n) can be expressed with eq. 1.7;

$$C_0 = n/R_s t \qquad (1.7)$$

where R_s is the sampling rate for the target analyte and t is the sampling time. The inter-sample matrices differences in semisolid tissues (such as fish muscle) are slight between individuals of the same species. Consequently, the sampling rate of the SPME fibre can be pre-determined under laboratory conditions and directly used for real sample analysis.[59] With this method, no K value determination and deuterated standard uploading prior to sampling is needed any longer. In this thesis, deuterated 2-MIB shared most of ions with 2-MIB in mass spectrameter, and limited the use of the on-fibre standardization calibration method. Therefore, 2-MIB determination followed this technique.

1.2.4 SPME device and coating

1.2.4.1 SPME fibre device

The main types of SPME devices applied during *in-vivo* sampling include fibre SPME, blade SPME and thin-film microextraction. When considering *in-vivo* sampling in fish, fibre and blade SPME are most widely used format. Due to the volatility of geosmin and 2-MIB, thermal desorption in GC is suitable for analysis. However, blade SPME coupled with GC is not a well-developed technique yet, making fibre SPME the best option for *in-vivo* sampling of volatile and semi-volatile compounds in fish. A typical commercial SPME fibre device from Supelco is shown in Fig. 1.5. The assembly contains a piercing needle and an inner tubing with a piece of coated fibre attached to it. Initially, SPME device designs utilized fibre cores made of fused silica or quartz. However, due to the fragility of such material, which can be easily broken during direct immersion in solid sample, metal and other alloy wires fibres were introduced in order to improve durability.

The key to keep the SPME device from leaking when it is inserted into a pressurized GC injection port is the sealing septem that seals the outer needle at the end.[37]

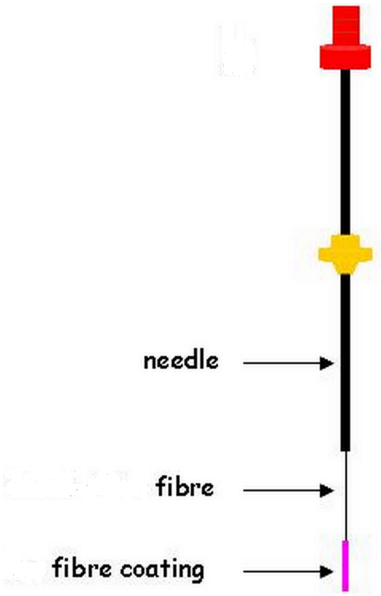


Figure 1.5 Commercial SPME device by Supelco[60]

1.2.4.2 Extraction phase

For direct *in-vivo* sampling in fish muscle, the extraction material must be biocompatible. From the SPME perspective, a biocompatible coating is one, which (i) does not cause toxic reactions to the system under study and (ii) does not permit strong adhesion of large biomolecules such as proteins to the surface of the coating.[37]

Currently, two commercial biocompatible SPME coating which can be coupled with GC are polydimethylsiloxane (PDMS) and poly (ethylene glycol) (PEG).[61] The performance of SPME is critically dependent on the properties of the extraction phase.

For example, the polarity of the coating decides the extraction phase selectivity while thermal stability and chemical inertness of the coating determines the sampling and analyzing conditions.[54]

Commercial PEG coating is not suitable for extracting geosmin and 2-MIB due to its polar characteristic. On the other hand, in literature, PDMS has been demonstrated as an effective extraction phase for geosmin and 2-MIB in fish. Indeed, Lloyd *et al*. reported that PDMS with thickness of 100 μm is effective for extracting geosmin and 2-MIB from fish.[46] Zhou *et al*. selected the PDMS fibre as the *in-vivo* SPME extraction phase of choice due to its better biocompatibility, and lack of competition or displacement effects compared with a solid coating.[56, 62] Besides, in a study by Jahnkea *et al*., it has been shown that PDMS can be used for passive sampling of non-polar, hydrophobic organic chemicals in heterogeneous and fatty tissues without its sorptive capacity being modified.[63] This conclusion is of great importance, since one of the greatest challenges for extraction from complex matrices is the fouling of the extraction phase. Using PDMS in the extraction phase, followed by a very simple step of fibre cleaning can keep fibre repeatable.

1.3 Binding between analytes and matrices

SPME can only extract freely dissolved analytes. For some SPME extractions, the amount extracted from an aqueous solution is negligibly small. In that case, the concentration on the fibre is linear proportional to the initial concentration.[64] However, biological samples such as fish muscle contain protein and lipids, which strongly bind

with geosmin and 2-MIB.[65] As a result, the amount of freely dissolved analytes available in the aqueous solution becomes limited, according to eq. 1.8 below:

$$X + M \rightleftharpoons XM \tag{1.8}$$

assuming that X is the analyte, which binds with matrix (M) in the sample under equibilibrium. If non-negligible extraction happens, the equibilium above will be disturbed, and shift to a new equilibrium. Under this situation, the extraction is not freely dissolved analytes only; added to it is the amount dissociated from binding matrix. On the contrary, if the extraction amount by the SPME fibre is very small, the equlibrium between X and M can be kept virtually undisturbed, and only the original freely dissolved chemical will be extracted by the SPME fibre.[64]

1.4 Objectives of the project

The presence of geosmin and 2-MIB in fish raised in RAS has been studied for a long time by researchers, and to date, there are still questions regarding: (1) the identification of microorganisms; (2) control of the key environmental, nutritional and operational parameters involved in the development of microorganisms responsible for the appearance of off-flavor compounds; and (3) the treatment of water to eliminate off-flavors. Therefore, developing a technique for on-site non-destructive detection of geosmin and 2-MIB *in-vivo* that involves sampling from individual fish repeatedly can facilitate the monitoring of any changes inside fish, as well as the changes in the environment.

The objective of this project was to develop an effective and simple method to determine geosmin and 2-MIB in fish using *in-vivo* sampling technique. In order to

accomplish this goal two kinetic calibration methods, including on-fibre standardization and using pre-determined sampling rates of analyts were investigated and verified. In addition, the binding effect between the analytes and the matrices was studied. Finally, the developed methods were applied for the on-site analysis. Results were compared to the one obtained from the traditional methods.

2 Chapter 2- Development of on-fibre standardization calibration method

2.1 Introduction

When a SPME liquid coating fibre preloaded with a standard is exposed to a sample matrix containing target analytes, desorption of the standard from the fibre and absorption of the analyte to the fibre occur simultaneously.[57] Analyzing it from the desorption side, the analyte diffuses through the boundary layer, which between the fibre surface and the bulk of the sample matrix, into the bulk of the sample matrix, while the absorption process performs the opposite direction against the desorption one (as shown in Fig. 1.4.).

The theory of on-fibre standardization was introduced in section 1.2.3.2. The isotropy between absorption and desorption can be proved using time profiles. If the sum of n/n_e and Q/q_0 is close to 1, the isotropism is verified.[56] Fig. 2.1 shows one example reported by Zhao *et al*. about the isotropy relationship of the absorption process of benzene and toluene, and desorption of deuterated benzene and toluene.

Another critical value to be taken into consideration was the amount of standard to be preloaded onto the fibre coating. Currently, four standard loading approaches are normally used, dependent on the volatilities of the compounds being analyzed: (a) headspace extraction of standard dissolved in a pump oil or direct extraction from a standard solution--for volatile compounds; (b) extracted from headspace of pure standards in a vial--for semi-volatile compounds; (c) direct spiked standard solution onto the fibre--for low volatility compounds.[66]

In this chapter, the development of deuterated standard uploading methods and fibre clean-up procedure after sampling from the fish tissue was developed, and the isotropy of desorption and absorption processes was verified.

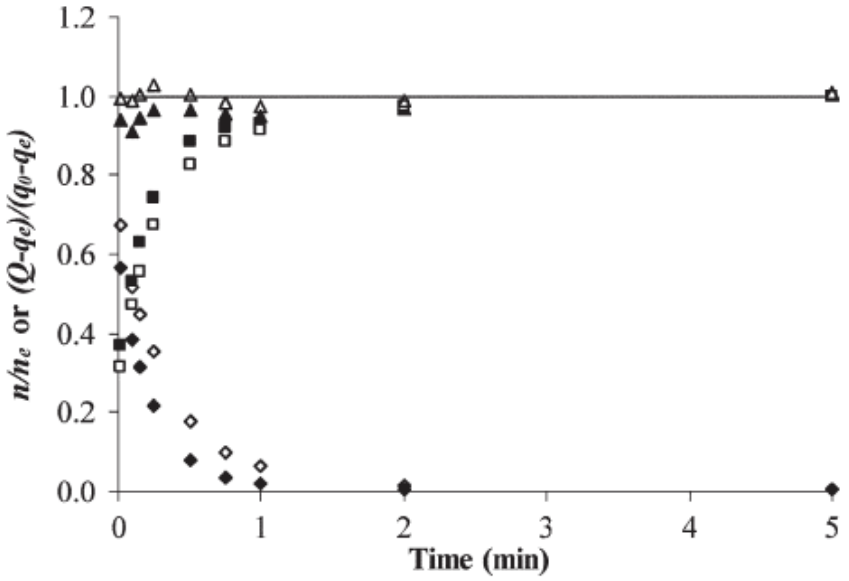


Figure 2.1 Isotropy of absorption and desorption in SPME. Simultaneous absorption of benzene (n) and toluene (□) and desorption of benzene-d_6 (u) and toluene-d_8 (◇); (▲) and (Δ), the sum of n/n_e and $(Q-q_e)/(q_0-q_e)$ for benzene and toluene, respectively.[3]

2.2 Experimental section

2.2.1 Chemicals and materials

Geosmin and 2-MIB were purchased from Wako Chemicals USA, Inc. Deuterated MIB (d_3-MIB) was obtained from CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada), and deuterated geosmin (d_3-geosmin) was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). All chemicals purchased were of the highest possible purity and were used without further purification. HPLC (high performance liquid chromatography) grade

methanol was purchased from Fisher Scientific (Unionville, Ontario, Canada). HPLC grade acetone was obtained from Caledon Laboratories LTD. (Georgetown, Ontario, Canada). Pump oil was purchased from Varian Vacuum Technologies (Lexington, MA). Triton X-100 was obtained from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Nanopure water was obtained using the Barnstead Nanopure water system. The 1 cm silica and metal core commercial PDMS fibres were obtained from Supelco (Bellefonte, PA). Taint-free rainbow trout fillets were purchased from Sobeys Inc. (Ontario, Canada). Rainbow trout fillets contaminated with geosmin and 2-MIB were obtained from Alma Aquaculture Research Station (University of Guelph, Ontario, Canada).

2.2.2 Method development of internal standard uploading procedure

Due to the absent amount of d_3-geosmin, all preliminary experiments were performed with d_3-MIB for method development of the preloading process. Moreover, because of the similar physical and chemical properties of these two compounds, it is reasonable to use only one compound. Four different uploading methods were compared: (a) headspace sampling from d_3-MIB dissolved in methanol solution; (b) headspace sampling from d_3-MIB pure solid; (c) headspace sampling from d_3-MIB dissolved in pump oil; (d) direct transfer 1 μL d_3-MIB methanol solution from the syringe to the fibre.

Another experiment was then performed based on extraction parameters from headspace of d_3-MIB and d_3-geosmin dissolved in a pump oil solution, where an experimental design was utilized. The purpose of it was to pursue the optimal conditions of the final uploading procedure. During this process, a three-factor, two-level full

factorial experimental design based on stir bar agitation speed, desorption temperature and desorption time inside the injection port was performed.

2.2.3 Fouling of the fibre

Two potential complications are typically observed when analytes are extracted from complex matrices. One is associated with competition among different compounds in the fibre and the other is the fouling of the extraction phase, due to the adsorption of macromolecules such as proteins and lipids at the interface.[39] These two issues limit the reproducibility and repeatability of the same fibre sampling. For PDMS coating, displacement due to competition in the coating is not as problematic as that observed in solid coating fibres.[54] However, previous research has identified the possibility of SPME fibre coating fouling taking place within biological and environmental samples associated with complicated matrices.[67-71]

Quite the reverse, Jahnke *et al*. reported that when SPME fibres with 30 μm PDMS coating were immersed in 15 different matrices, including very complex samples such as fish tissue for non-polar, hydrophobic organic chemicals extraction, then paired with a very simple wipe clean step, the sorptive properties of PDMS remained largely unaffected. [63] Wang *et al.* also reported that using the dry wiping step with Kimwipe™ could easily clean the fibre without any properties changes.[72]

In the present study, fouling potential was investigated through repeated extraction from headspace of the uploading generator vial using tissue-treated and nontreated fibres (details in section 2.3.2).[73] All fish tissue experiments were performed

in accordance with protocols approved by our institutional Animal Care Committee (AUP No. A-12-01) in the Chemistry Lab Facility at the University of Waterloo.

2.2.4 Isotropy of desorption and absorption of geosmin and 2-MIB in fish muscle

Isotropism was verified by performing desorption time profiles of deuterated standards and absorption time profiles of analytes simultaneously. Both homogenized and non-homogenized fish tissue samples were utilized. From eq. 1.5, n_e, which is the extraction amount under the equilibrium condition, has to be determined. Contaminated homogenized fish muscle was obtained by spiking specific amounts of geosmin and 2-MIB methanol solution into taint-free homogenized fish tissue. Non-homogenized fish tissue with contaminatants was prepared by cutting each sample in 4-g size. Sampling was conducted by directly inserting the SPME fibres in the homogenized and non-homogenized fish tissue. All the experiments were performed under 8.5 °C, the same temperature as *in-vivo* sampling in fish.

2.2.5 Instrumentation

An Acme 6000 Series gas chromatography flame ionization detector (GC-FID) (Korea) was used for deuterated standard uploading procedure optimization. The GC was equipped with a split/splitless injector and a capillary column (RTX-5, 30 m × 0.25 mm I.D., 0.25 μm film thickness). The GC column oven temperature programm was based on the results obtained by previous colleagues: 60 °C (0.5 min) ⇒ 40 °C/min to 110°C ⇒ 5

°C/min to 140 °C ⇒ 40 °C/min to 250 °C (10 min). The column flow rate of the helium carrier gas was 1 mL/min. The temperature of the injector was 250 °C.

The isotropy verification experiment was achieved with Varian 3800 GC coupled with Varian 4000 electron ionization ion-trap mass spectrometer (GC-EI-IT-MS). The split/splitless injector was equipped with a Merlin microseal injector adapter (Merlin Instrument Company, Half Moon Bay, CA, USA) for metal fibre desorption. The type of capillary column and the column flow rate of helium carrier gas were set for the same as Acme GC-FID mentioned above. The 1079 injector was equipped with a programmed temperature vaporizer. The oven temperature programm was 60 °C (0.5 min) ⇒ 40 °C/min to 110°C ⇒ 1 °C/min to 117 °C ⇒ 5 °C/min to 156 °C⇒ 40 °C/min to 250 °C (10 min). The temperature of the transfer line was 280 °C and 240 °C for the ion trap. Selected ion storage (SIS) mode was used for scanning geosmin, d_3-geosmin, 2-MIB and d_3-MIB.

2.3 Results and discussion

2.3.1 Preloading procedure determination

The amount of standards preloaded onto the fibre should be at a level that is not too high when compared to the analyte extraction amount at sampling time *t*, and not as low as to cause detection problems. An effective uploading procedure should be fast and reproducible. The same standard generator vial can be used for hundreds of loadings.[66]

Headspace sampling from methanol solution. Headspace, when compared with direct immersion can reduce the chance of fibre deterioration. However, due to the

volatility of methanol, a large amount of the solvent had to be extracted by the fibre coating, which overlapped the analyte peaks.

Headspace sampling from pure standard. Even 1 mg pure solid d_3-MIB in a 20 mL vial with 5 s extraction time under room temperature could cause peak overloading. The vapor pressure of 2-MIB is too high.

Headspace sampling from pump oil solution. As mentioned previously, pump oil can significantly reduce the amount of standards in the vial headspace due to the lower distribution coefficient that exists between the headspace and the pump oil.[55] An amount of 2 mg d_3-MIB was dissolved in 10 g pump oil in a 20 mL vial. The extraction temperature was kept at 25 °C. The vial, equipped with a stir bar inside, was agitated with a speed of 1600 rpm. With this approach, each loading cycle of 30 s extraction withdrew only 0.00009 % of d_3-MIB from the standard generation vial, which means that the same vial can be re-used for thousands of times without significant concentration depletion. The reproducibility observed for 15 times extraction under 30 s was with a RSD (relative standard deviation) 1.8 %. The amount of standard loaded onto the fibre could be easily adjusted by either changing the initial concentration in the pump oil or the extraction time.

Syringe-fibre transfer. This method was performed by loading d_3-MIB methanol solution onto the fibre. After the evaporation of solvent, the fibre was injected into GC-FID for seperation and quantification. Compared to the direct injection of 1 μL 100 μg/mL standard solution to the GC, the d_3-MIB amount left on the fibre coating was only around 60 % due to the evaporation of d_3-MIB. Moreover, compared to extraction from headspace of pump oil, this method was more complicated.

Table 2.1 Three-factor two-level full factorial experimental design

Exp. No	A(rpm)	B(min)	C(°C)	amount y1(ng)	amount y2(ng)	Average extraction amount y (ng)
1	200	1	250	7.21	7.02	7.11
2	1500	1	250	7.05	7.11	7.08
3	200	5	250	7.09	6.71	6.90
4	1500	5	250	6.81	7.03	6.92
5	200	1	280	7.10	7.57	7.33
6	1500	1	280	7.38	7.63	7.50
7	200	5	280	7.21	7.64	7.42
8	1500	5	280	7.12	6.95	7.04

Comparison of the above experiments, headspace sampling from pump oil is the most suitable approach for the preloading the deuterated geosmin and 2-MIB standards. In addition, in order to achieve optimal preloading results and well maintain instrument, a three-factor and two-level experimental design was utilized. It is well known that an increase in the concentration of standard solution and time of extraction will cause an increase in the extraction amount on the fibre. Also, apart from the above-mentioned conditions, other conditions may have an influence on the uploading procedure. For example, stir speed, desorption time and desorption temperature inside the injector are potential significant factors. (Table 2.1) Results were calculated with matrix algebra. However, the coefficients of all effect were far smaller than three times of the standard error, meaning these factors all exhibited insignificant effect on the extraction amount. For better maintenance of instrument and to minimize time spent, the final extraction

condition was set as: 200 rpm stir speed and desorbed for 1 min under 250 °C in GC injector.

2.3.2 Optimization of fibre-washing step

Fouling of fibre was determined by evaluating the reproducibility of the fibre after repeating exposed to the fish tissue. After tissue treated, the fibre was used to extract from the headspace of the deuterated standard solution, and the RSD % of the extracted amount was compared to the non-tissue treated fibre. The purpose of this approach was to eliminate the variation of analytes existing in the fish sample. The procedure for this process begins by inserting SPME fibre into uncontaminated fish fillet for 30 min, followed by washing the fibre under a small stream of water with wash bottle, then wiping the fibre with Kimwipe. After that, a 30 s extraction from headspace of d_3-MIB and d_3-geosmin in pump oil solution was performed to inspect if there is a change in the extracted analytes due to fouling after repeats. Fig. 2.2 shows that fouling did have an impact on the fibre in the form of enhancement after four repeats. The RSDs were 9.4 % for d_3-MIB and 6.4 % for d_3-geosmin. Compared with 1.8 % and 1.2 % for untreated fibre, it confirms that biofouling disturbed the analytes mass extraction. In addition, an obvious carryover was observed on the fibre after four injections. Due to the relative high cost of SPME fibre, a washing step had to be implemented in order to accomplish repeatable utilization of fibre.

Although fibre fouling is a common issue found in complex sampling with SPME, seldom do studies report approaches used for fibre washing. Normally, wiping with Kimwipe or water stream followed by dry wipe is the standard procedure for fibre

cleaning. The reason biofouling occurs is the adsorption of macromolecular compounds, such as proteins and lipids on SPME coating. Unlike solid coating, all the big molecular compounds are accumulated on the surface of PDMS coating instead of in the pores of solid coating, which results in possibility of completely recovering the PDMS coating.

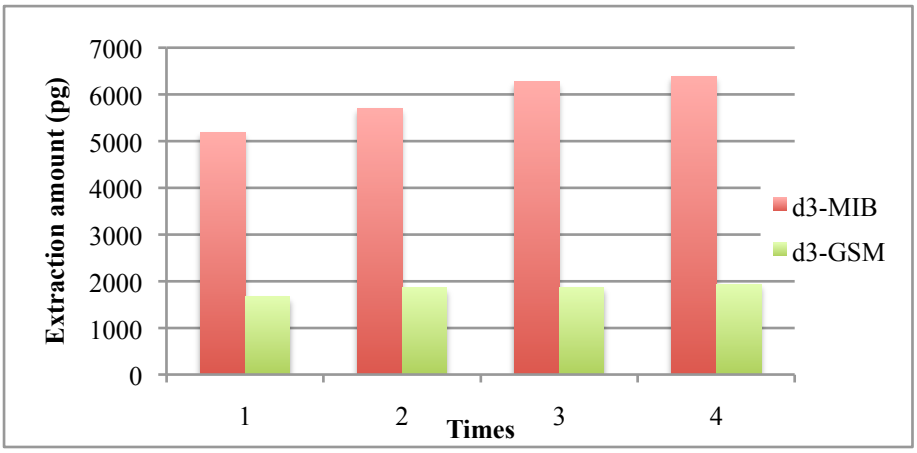


Figure 2.2 Biofouling confirmation with tissue-treated fibre

Cleaning the fibre with methanol. Dip washing in methanol has been investigated by other colleagues after sampling from the grapes and results demonstrated the effectiveness of this washing step. However, when following these steps on fish sampling, the black dots found on the coating, which might be caused by high temperature oxidization of proteins and lipidsin the GC injector. Fortunately, a bit of force stressed, while at the same time wiping the fibre with methanol was found to effectively remove the black dots. However, a series of fibre test experiments based on the same tissue-treated fibre as above followed by a methanol wiping step indicated that forced wiping would cause surface damage of PDMS coating. As shown in Fig. 2.3, the first six extractions were kept at an acceptable repeatability, with RSDs values being 3.9

% and 3.3 % for d_3-MIB and d_3-geosmin, respectively. However, once past the sixth extraction, an obvious drop of extraction amount led to RSDs 9.0 % for d_3-MIB and 5.8 % for d_3-geosmin. Pictures taken under microscope (Fig. 2.4) indicated that the forced wiping procedure deteriorated the coating surface.

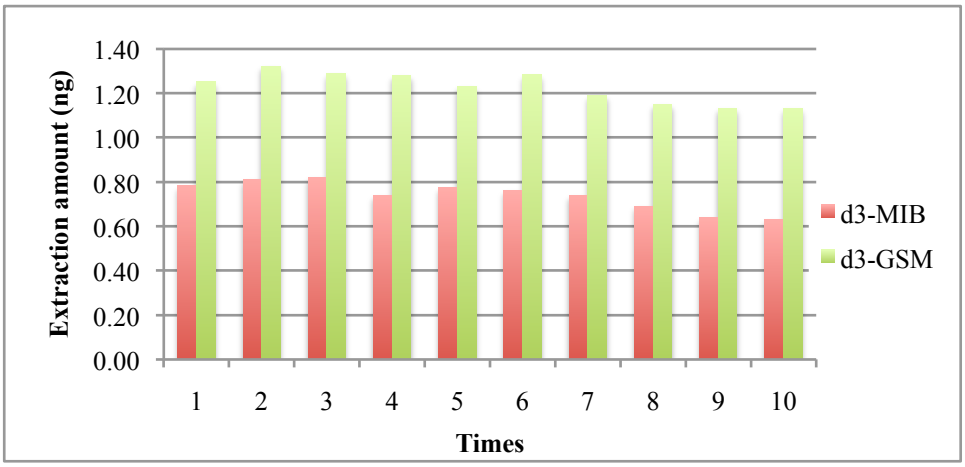


Figure 2.3 Extraction amount with methanol wiping step

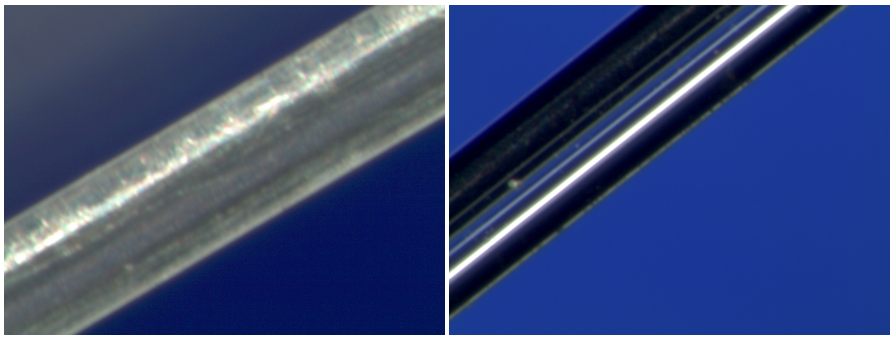


Figure 2.4 Left image shows the rough surface of fibre after wiping with methanol for ten times; right image is the picture of a brand new fibre

Clean the fibre with Triton X-100 water solution. Triton X-100 is a commonly used detergent in laboratories.[74, 75] A 1 % (w/w) Triton X-100 water solution was prepared for fibre cleaning. After washing the fibre with Triton X-100 solution, the fibre was then rinsed with a large amount of water to avoid any detergent leftover. However, black dots on the coating were found as same when using methonal dip washing procedure, and caused carryover problem.

Clean the fibre with Acetone. A large amount of fatty acid information could be observed through mass spectrum, meaning lipids may indeed play an important role in fibre fouling. It is a well-known fact that lipids can be easily dissolved in non-polar solvents due to similar polarity. By considering toxicity and cost, acetone was chosen as the fibre washing solvent. The procedure used was as follows: after extraction from the fish sample, the fibre was then (a) washed with a small stream of nanopure water from a wash bottle; (b) gently wiped by Kimwipe; (c) injected into GC injection port for desorption; (d) gently wiped with acetone soaked Kimwipes; (e) rinsed with large amounts of water; (f) conditioned in GC injector for 10 min at 250 ºC. Repeated testing of this approach was done by using tissue-treated fibre 11 times, with satisfactory results obtained. (Fig. 2.5) Fig. 2.6 shows an image captured by microscope after 37 extractions with the aforementioned washing step, showing the surface of PDMS coating was still smooth. In this experiment, the RSDs for d_3-MIB were 4.2 % and 4.6 % for d_3-geosmin.

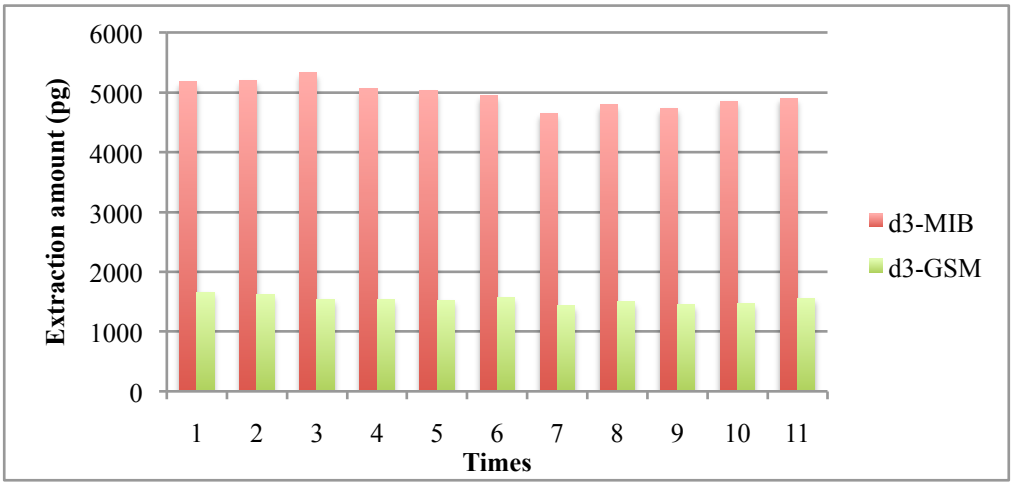


Figure 2.5 Repeatability test using acetone-wiping procedure

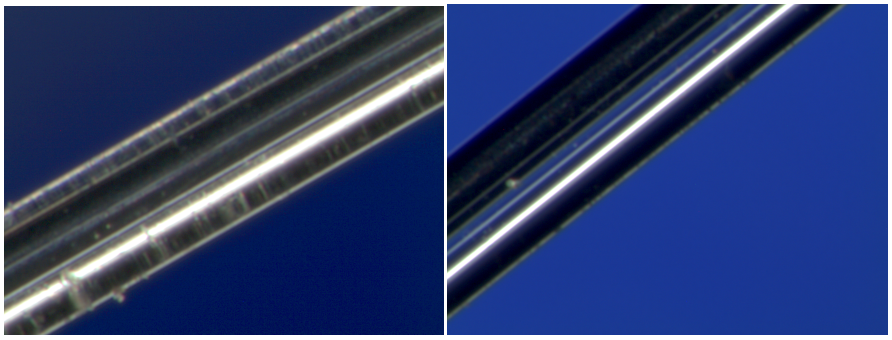


Figure 2.6 Left image is the fibre surface after using acetone-wiping approach 37 times; Right image shows the picture of a brand new fibre

2.3.3 GC temperature time programming optimization

After optimizing the fibre-washing step, no matter how to clean the fibre using washing step, one peak always coeluted with 2-MIB. From the MS library, the compound should be a fatty acid, which could either come from fish tissue, the vial or even the experimental gloves.[46-48] The current GC temperature time programming was optimized

by colleagues, and the method was found time-efficient and well selective, with the exception of the coeluting problem mentioned above.

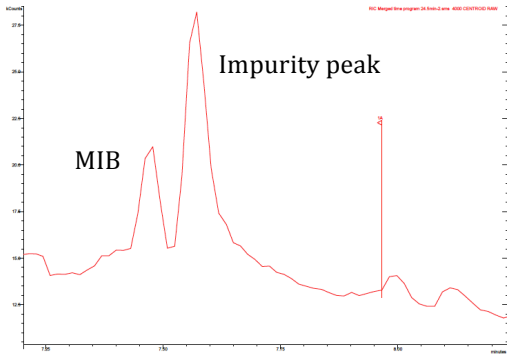


Figure 2.7 Successful separation of 2-MIB and the contaminatant

Therefore, optimization based on previous programming could be achieved in order to separate the contamination peak and 2-MIB. The first effort was made by reducing the temperature-raising rate from 5 ºC/min to 3 ºC/min. In it, it could be observed that the peak of 2-MIB was separated from contamination peak, however, there was still overlap observed between them. Ultimately, a 1 ºC/min rate was chosen instead of the 5 ºC/min rate previously used. (Fig. 2.7) The total analysis time became 28.9 min, just 3 min longer than the previous one.

2.3.4 GC-MS ions selection

Selected ion storage (SIS) mode was utilized for isotropy determination. To analyze geosmin, d_3-geosmin was used as an internal standard. In this case, the EI spectrum of the labeled compound produced a base peak at *m/z* 115, which contained

three deuterium atoms, in the agreement with the fragmentation proposed by Lloyd *et al.*[78] Eventually, the quantification of geosmin and d_3-geosmin was achieved using the ion pair at *m/z* 112 and 115, and qualification was established by using the ions *m/z* 126 and 182 for geosmin and *m/z* 129 and 185 for d_3-geosmin.

To separate 2-MIB and d_3-MIB, the base peak ion at *m/z* 95 could not be used, since it is shared by both compounds, which are not chromatographically resolved. (as shown in Fig. 2.8) Indeed, not a lot of detailed information is available on how to separate 2-MIB and labeled 2-MIB using MS. Palmentier *et al*. used the ions at *m/z* 150 and 153 for quantification in their analytical method.[79] However, they pointed out that high-resolution mass spectrometry was required in this case, because the signal at *m/z* 153 originated from both d_3-MIB and 2-MIB and the *m/z* 153 ions from each compound are indistinguishable at low resolution.[80] McCallum *et al*. chose a "soft" chemical ionization (CI) technique using *m/z* 151 and 154 to achieve considerably enhanced sensitivity relative to that achieved by using EI, with molecular ions at *m/z* 168 and 171. However, it is impossible to perform CI in our case.

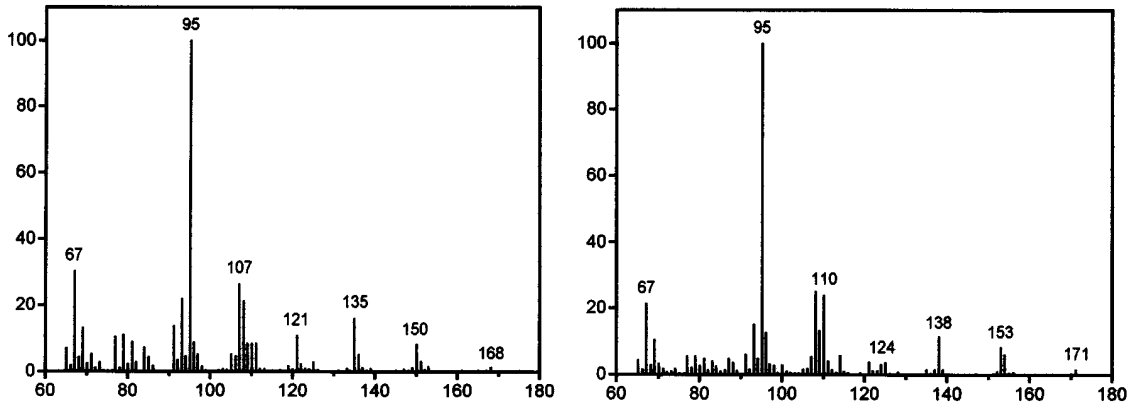


Figure 2.8 EI-mass spectrum of 2-MIB (left) and d_3-MIB (right) [80]

While with further inspection of the spectrum in Fig. 2.8 showing that there were more intense ion pairs than molecular ions that could be used for quantification. Finally, *m/z* 150 and 138 were decided to represent 2-MIB and d_3-MIB as quantification ions, the two not being common to each other. Likewise, *m/z* 168, and *m/z* 171 were decided as qualifiers for 2-MIB and d_3-MIB, respectively.

2.3.5 Isotropy of desorption and absorption verification

The kinetics of absorption and desorption was performed in both homogenized fish tissue and non-homogenized fish tissue in order to validate isotropy of desorption and absorption process.

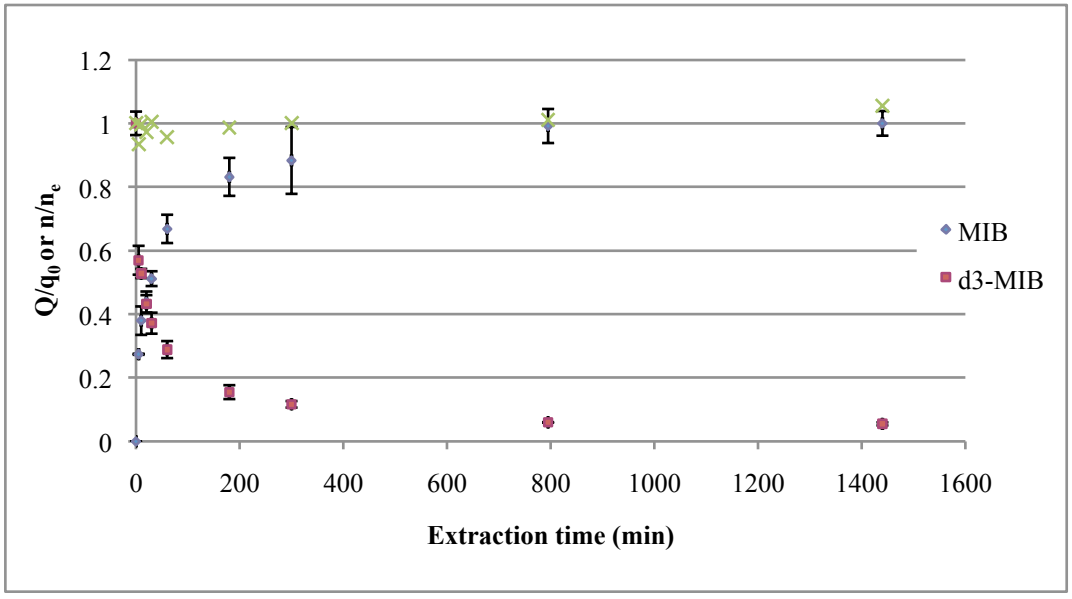


Figure 2.9 2-MIB absorption (◆) and desorption (■) time profiles. The absorption was performed in homogenized fish tissue containing 0.5 µg/g 2-MIB and 0.025 µg/g geosmin in a 2 mL GC vial under 8.5 °C. For desorption profile, the standards were preloaded from the headspace of 200 µg/g d_3-geosmin and d_3-MIB pump oil solution under 25 °C for 30 s. 100-µm PDMS metal core fibres were used to produce the profiles. **(✕)** is the sum of n/n_e and Q/q_0.

Time profiles were used for this verification. The absorption time profile was obtained by using the same concentrations of geosmin and 2-MIB in the sample but different absorption times for each point. On the other hand, desorption time profile was drawn by preloading same amounts of analytes but different desorption time for each point. The sum of n/n_e and Q/q_0 was close to 1 for both 2-MIB and geosmin in non-homogenized and homogenized fish tissue, which means the symmetry was kept all through the desorption and absorption process. The small deviation of the sum from 1 can be ascribed not only to the difference of physicochemical properties between deuterated standards and non-deuterated ones, but also the experimental errors introduced by a complex sample matrix. Fig. 2.9 sets 2-MIB as an example to show the symmetry between desorption and absorption in homogenized fish tissue.

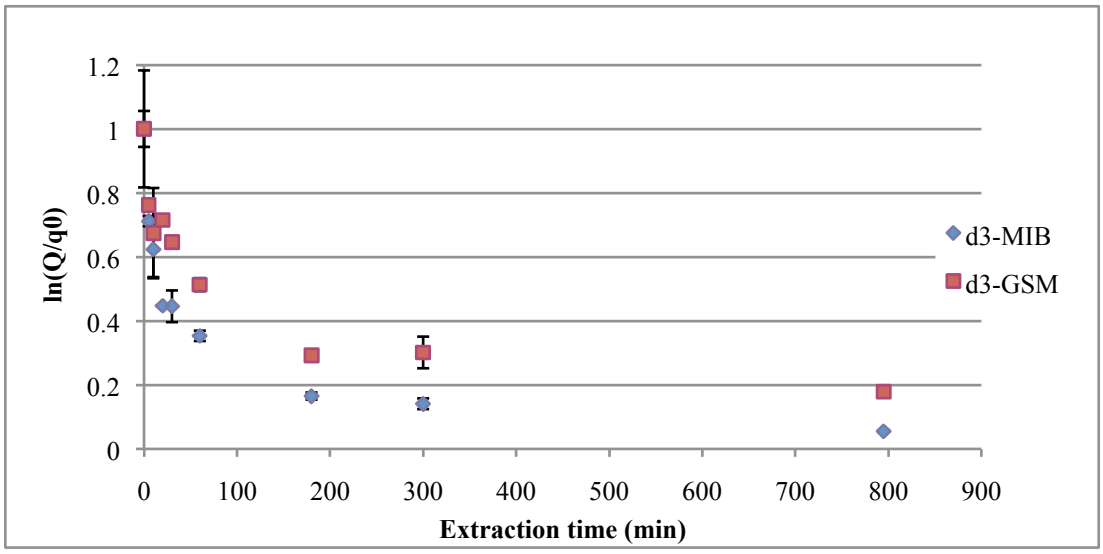


Figure 2.10 Desorption time constant profiles of d_3-MIB and d_3-geosmin in non-homogenized fish tissue

The time constant profiles can be obtained with ln (1-n/n_e) or ln (Q/q_0) as the y-axis, and t as the x-axis, where the regression slope is $-a$, as shown in Fig. 2.10. Unlike Zhou *et al.*'s results,[2] in which time constant profiles were kept linear from the very beginning of extraction process until equilibrium, in this experiment time constant profiles only stay linear at the first 5-60 min extraction time (Fig. 2.10, Fig. 2.11). For both analytes the time constant profiles lost linearity after 60 min.

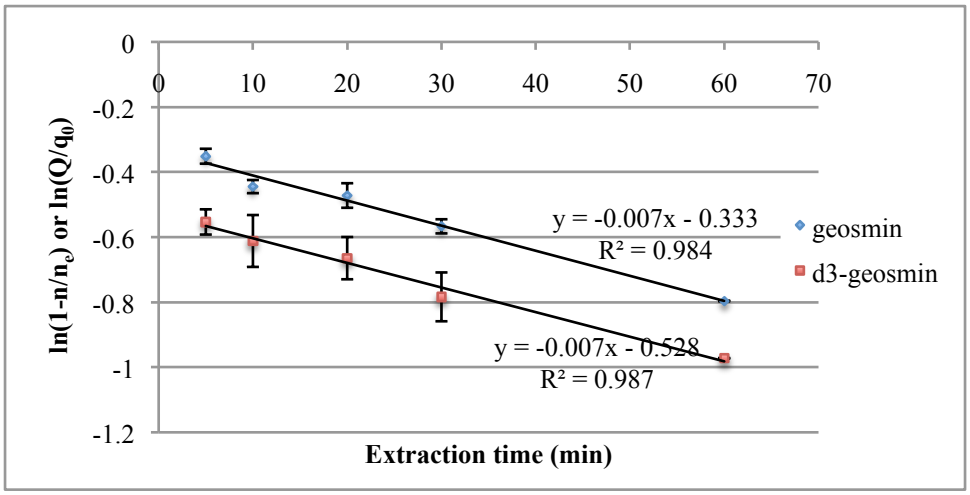


Figure 2.11 Time constant profiles of the linear part of d_3-geosmin and geosmin in homogenized fish tissue

In Chen *et al.*'s study, it is indicated that when using on-fibre standardization method, exposure of the fibre to a sample matrix just one time point is enough to determine the time constant a.[57] However, since the linearity of both analytes within the first 5-60 min were observable, at least five points could be selected to form a straight line and obtain the slope to get the time constant. In this case, the time constants are more reliable and robust. Table 2.2 shows the time constant results obtained within the linear

part, with five time points (5, 10, 20, 30 and 60 min) being utilized. Linear regression for homogenized fish tissue was better than that for non-homogenized fish tissue due to the inconsistent compositions in different parts of fish muscle.

Table 2.2 Time constant results and conclusions for 2-MIB and geosmin in homogenized fish tissue and non-homogenized fish tissue

Homogenized fish tissue					
2-MIB	Desorption *a* (/min) (R^2)	Absorption *a* (/min) (R^2)	Geosmin	Desorption *a* (/min) (R^2)	Absorption *a* (/min) (R^2)
	0.012±0.002 (0.946)	0.011±0.001 (0.984)		0.008±0.001 (0.987)	0.008±0.001 (0.984)
Non-homogenized fish tissue					
2-MIB	Desorption *a* (/min) (R^2)	Absorption *a* (/min) (R^2)	Geosmin	Desorption *a* (/min) (R^2)	Absorption *a* (/min) (R^2)
	0.012±0.003 (0.844)	0.012±0.006 (0.780)		0.007±0.001 (0.884)	0.010±0.004 (0.837)

2.4 Conclusion

An optimized uploading procedure was decided with extraction from headspace of deuterated standards in pump oil under 25 °C. With this procedure, more than thousands of times sampling can be performed in the same vial without concentration depletion. The preloading amount can be adjusted by changing the concentration of standards in the solution or changing the extraction time. Biofouling was solved by gently wiping the fibre with acetone soaked Kimwipe after desorption. Quantification and qualification of

non-deuterated and deuterated geosmin and 2-MIB in MS were investigated. Ions to represent 2-MIB and d_3-MIB for on-fibre standardization were chosen at *m/z* 150, 168 and *m/z* 138, 171, respectively; *m/z* 112, 126, 182 and *m/z* 115, 129, 185 were selected for geosmin and d_3-geosmin, respectively. The sysmmetry relationship between geosmin and 2-MIB desorption and absorption processes in homogenized and non-homogenized fish were verified. This means that the on-fibre standardization calibration method is applicable for determining the concentration of 2-MIB and geosmin in fish samples. The time constants of 2-MIB and geosmin for rainbow trout in the first 5-60 extraction time under *ex-vivo* condition were 0.0012 /min and 0.008 /min, respectively, and the differences found in the time constants were kept within standard deviation as different fish utilization occurred.

3 Chapter 3- Does binding between fish matrices and off-flavor compounds affect SPME measurement?

3.1 Introduction

Freely dissolved concentration measurements using SPME under non-equilibrium conditions have been investigated by a number of researchers.[81-83] However, in order to properly analyze off-flavor compounds in fish, the concentration measurement should be more biased towards total concentration, since the high temperature cooking treatment of fish before consumption can release the bound analytes to the freely dissolved ones. Zhao has investigated the binding effect in aqueous samples containing humic organic matter in her Ph.D. thesis for SPME total concentration measurement.[84] While compared to aqueous samples, fish matrix as a static solid condition may exhibit a different kinetic process. Although the usage of kinetic calibration SPME to determine the total concentration of analytes in fish has been studied,[62, 73, 85, 86] it is seldom discussed how binding constitutes may affect the extraction process. Explaining the kinetic process of total concentration measurement can be determined based on the theory proposed on the binding effect of free concentration determination.

Extraction of freely dissolved concentration in samples containing a matrix that binds to the compound of interest requires two conditions to be met. First, the freely dissolved analytes should not be depleted by the SPME extraction.[81, 82, 87] Secondly, matrices in a sample may not interfere with the analyte uptake onto the fibre.[88] For most SPME extractions, the sample size is much greater than the volume of the extraction phase. In this case, the depletion of analytes inside the sample is negligible. In Vaes *et al.*'s study, they concluded that with negligible depleted SPME (nd-SPME), leaving the

freely dissolved concentration virtually constant, all binding equilibria remain undisturbed.[82] The definition of negligible SPME is introduced differently in different sources. In Pawliszyn's book, below 10 % percent depletion can be accounted as negligible depletion,[54] while Heringa *et al.* indicated a limit of 5 % should be considered a significant depletion level.[89] However, in some circumstances, the consumed amount of analytes is relatively small (<5 %) when compared to the total amount in the sample, but local depletion occurs in the boundary layer around the fibre.

The boundary layer is formed when an SPME fibre is introduced into a sample matrix, coming between the fibre surface and the bulk of sample matrices. When agitation of the sample is weak or static, diffusion in the boundary layer controls the overall mass-transfer rate.[57] (as shown in Fig. 3.1) Furthermore, it can be expected that only non-bound analytes diffuse into the liquid fibre coating. Local depletion is caused as a result of analyte uptake by the fibre; freely dissolved concentration in the boundary layer is later reduced. It follows that analytes sorbed to the binding matrix in the boundary layer can dissociate and subsequently contribute to the analyte flux toward the SPME fibre. As a consequence, equilibrium between the fibre and the sample is reached earlier than a sample without a matrix.

The binding effect under pre-equilibrium conditions like this has been reported by others previously, and one proposed solution to this problem is to perform all analysis using negligible equilibrium extraction.[88, 90, 91] In that case, there is no difference anymore in the fibre concentration in samples with and samples without binding matrix. However, while long equilibrium extraction time is a challenge for *in-vivo* sampling in live fish, pre-equilibrium extraction still should be investigated in details in order to

determine how binding affects the uptake process, as well as confirming whether depletion happens in such cases.

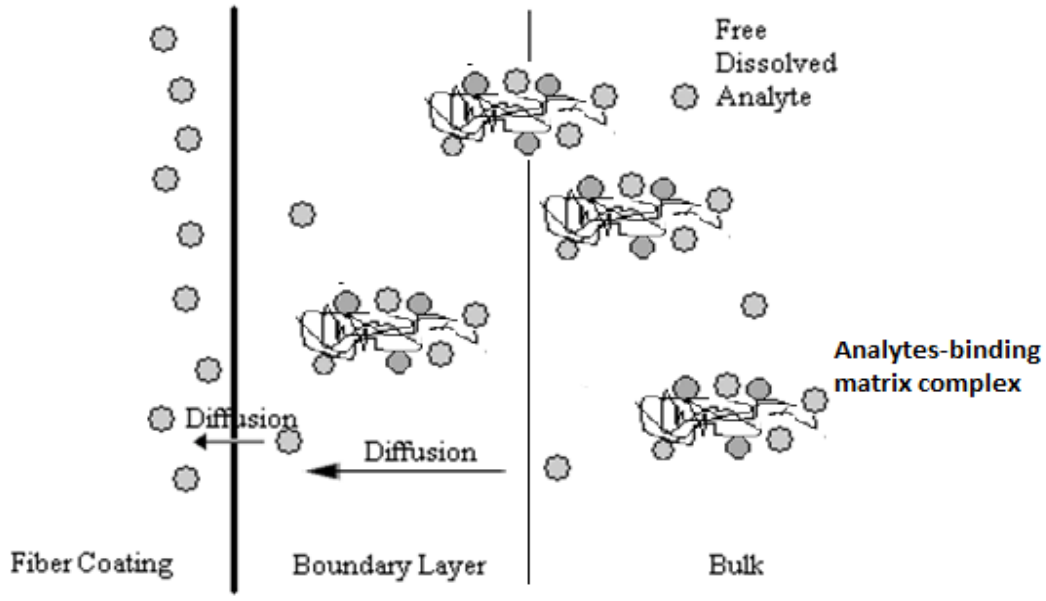


Figure 3.1 Conceptual representation of the uptake model for analyte fluxes toward the SPME fibre coating. Both the freely dissolved analytes and the sorbed analytes diffuse into the boundary layer

3.2 Experimental section

3.2.1 Chemicals and materials

All deuterated and non-deuterated standards, methanol, acetone, pump oil, PDMS metal core fibres and non-contaminated rainbow trout fillets were obtained in the same manner as described in section 2.2.1. Agarose was purchased from BDH Laboratory Supplies (Pooles, England).

3.2.2 Agarose gel model

As mentioned above, the movement of analytes from fish muscle to fibre is based on diffusion as a mass transfer process between fish tissue and fibre. Thus, diffusion parameters such as mass transfer coefficient (diffusion coefficient), as well as absorption and desorption rates are all important parameters when examining the extraction process. Agarose gel is currently being used to study the diffusion process of analytes, since without binding matrix, it could deliver the information about the freely dissolved analytes.[85] Moreover, due to its similar semisolid and permeable structure, agarose gel has been widely used as a medium to simulate animal tissue in the investigation of diffusion mechanisms.[92] As a result, agarose gels of different tortuosity (details in section 3.3.1) were compared in order to find a gel model most suitable to mimic fish tissue while observing the kinetics of 2-MIB and geosmin.

3.2.3 Sample preparation

Different concentrations of binding matrices were prepared by mixing different ratios of taint-free homogenized rainbow trout tissue with liquid agarose gel, to make a 2 g sample. Stock methanolic solutions of geosmin and 2-MIB were spiked into 2 mL GC vial prior to fish and gel addition. Next, the fish-gel solution was agitated for 1 min, and then allowed to come to analyte and matrices equilibrium overnight. Following, direct immersion SPME extraction was applied. All the experiments were performed under 8.5 ºC, which was the same temperature as in RAS.

Homogenized fish samples containing analytes were prepared by spiking geosmin and 2-MIB methanol solutions into taint-free homogenized tissue. A severe mixing round followed in order to make sure the concentration in each part of the sample was equal. After that, homogenized samples were distributed into 2 mL GC vials with amount of 2 g.

3.2.4 Instrumentation

The instrumentation used in this analysis was the same as in section 2.2.5, Varian 4000 Ion trap coupled with Varian 3800 GC. The optimized oven temperature programming was 60 °C (0.5 min) ⇒ 40 °C/min to 110°C ⇒ 1 °C/min to 117 °C ⇒ 5 °C/min to 156 °C⇒ 40 °C/min to 250 °C (10 min). SIS scan mode was used for all the experiments. The selected isotropy experiment quantification ions for each compound were: *m/z* 112 for geosmin, *m/z* 150 for 2-MIB*, m/z* 115 for d_3-geosmin, and *m/z* 138 for d_3-MIB. As qualifier ions, *m/z* 126 and 182, *m/z* 168, *m/z* 129 and 185, and *m/z* 171 were selected for the confirmation of each analytes identity. Except for isotropy experiments, as the common ions issue of 2-MIB and d_3-MIB, other experiments, which did not require d_3-MIB in the system, were all performed with quantification ion *m/z* 95 and qualification ions *m/z* 107 and 168 for 2-MIB. All other parameters utilized were the same as in section 2.2.5.

3.3 Results and discussion

3.3.1 The most suitable gel to mimic fish tissue

The purpose of using agarose gel to mimic fish tissue is to set up a non-binding sample matrix, which can aid in understanding the relationship between total concentration and free concentration. In this experiment, the tortuosity of gel was dependent on the amount of agarose inside the phosphate-buffered saline (PBS) solution. The selection of 0.9 % gel was made previously in another study to simulate fish for pharmaceutical analysis with SPME.[85] In current experiment, 0.5, 0.8, 1.0 and 2.0 % (w/w) agarose gel solutions were compared based on the evaluation of the desorption time constants of geosmin and 2-MIB. Table 3.1 shows the desorption time constants of 2-MIB and geosmin in different tortuosity of gel. All values were calculated using the slope of time constant profiles within 5-60 min (5, 10, 20, 30 and 60 min).

Table 3.1 Desorption rate constants of d_3-MIB and d_3-geosmin in different tortuosity of gel and homogenized fish tissue

d_3-MIB		d_3-geosmin	
Matrix	Time constant a (/min)	Matrix	Time constant a (/min)
0.5 % gel	0.016±0.001	0.5 % gel	0.014±0.002
0.8 % gel	0.014±0.001	0.8 % gel	0.010±0.003
1.0 % gel	0.012±0.001	1.0 % gel	0.008±0.003
2.0 % gel	0.009±0.001	2.0 % gel	0.006±0.001
Homogenized fish tissue	0.012±0.002	Homogenized fish tissue	0.008±0.001

Desorption rate as opposed to absorption rate was used in this case due to symmetry characteristic confirmed in section 2.3.5. In addition, desorption time constant could be obtained more easily due to the sample preparation procedure used in this case. Therefore, desorption time constant was used in this experiment. According to Table 3.1, 1% agarose in PBS (w/w) shows the same desorption constant as homogenized fish tissue regarding to both 2-MIB and geosmin, and was chosen to simulate fish tissue in the following study.

3.3.2 Confirmation of binding existing in SPME extraction process

3.3.2.1 Different concentration of binding matrices

All time constants were obtained within the first 5-60 min absorption or desorption time. The isotropy of desorption of deuterated standards from fibre and absorption of analytes onto fibre was confirmed with gel and 40 % fish tissue in gel, as shown in Table 3.2. Fish tissue in gel with values of 0 %, 10 % and 40 % were prepared in order to compare the time constants. Due to the symmetry confirmation of geosmin and 2-MIB in both gel and 40 % fish tissue in gel, it was concluded that 10 % fish tissue in gel would follow the same characteristic. Thus, only desorption time profiles for different concentration of binding matrices were compared. The results indicate that from 5-60 min, the increasing concentration of binding matrix does not have any impact on the shape of the desorption time profile, but only affect the desorption amount of analytes from the fibre. Raising the concentration of the binding matrices led to a larger amount of desorption. As shown in Fig. 3.2, Q in y-axis represents the amount of deuterated

geosmin left on the fibre after desorption. The uploading amount of standards before desorption were equal for all of them. However, due to the association of d_3-geosmin from the fibre with binding matrices in the sample, the quantity of desorbed compounds from fibre in higher concentration of binding matrices was greater than that in lower concentration. Therefore, an assumption can be made that the association between desorbed d_3-geosmin from fibre and binding matrices dominates the desorption process in the first 5 min, but afterward, binding matrix does not have any effect on the rate of desorption. The same phenomenon was observed in 2-MIB as well.

Table 3.2 Symmetry of 2-MIB and geosmin in gel and 40 % fish tissue in gel

Fish % in gel (w/w)	2-MIB		Geosmin	
	Desorption time constant *a* (/min)	Absorption time constant *a* (/min)	Desorption time constant *a* (/min)	Absorption time constant *a* (/min)
0%	0.012±0.001	0.012±0.001	0.008±0.003	0.009±0.002
40%	0.013±0.001	0.011±0.004	0.009±0.002	0.008±0.001

Moreover, absorption amounts are related to free concentration in the sample, which becomes lower when a higher concentration of binding matrix existed in the system. Both 0 % and 40 % samples had the total concentration of 1.0 μg/g for 2-MIB and geosmin, and, because of binding, 40 % fish tissue in gel obtained a lower extraction amount of analytes than 0 % fish tissue in gel under the same extraction time.

In addition, an interesting phenomenon was found by comparing the desorption and absorption time constant profiles of homogenized fish tissue and 1 % gel. In gel

system, the time constant profile kept linear all the process, which was unlike that of homogenized fish tissue, the linearity only remained in the first 5-60 min (as shown in Fig. 2.10). The details will be explained in section 3.3.2.3.

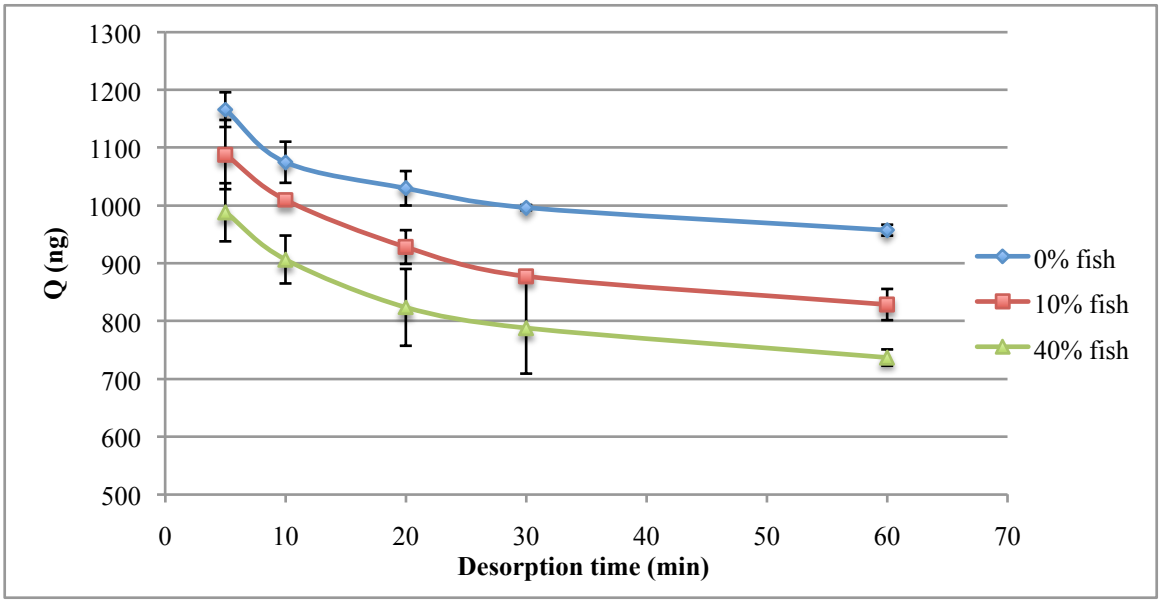


Figure 3.2 Desorption time profiles for d_3-geosmin of effect on different concentrations of binding matrices

Thus, the change of concentration of binding matrix does not seem to have an effect on the kinetics of geosmin and 2-MIB in the first 5-60 min of extraction.

3.3.2.2 Binding percentage determination

For this experiment, the same total concentration (50 ng/g) of gel and homogenized fish tissue were prepared. Equilibrium extraction was performed. The

percentage of binding was obtained with eq. 3.1. Gel was used to acquire total concentration, with the assumption of no binding matrix existing in it. As a result, binding percentage of 2-MIB and geosmin were determined to be 96.52 % and 99.23 %, respectively, meaning only 3.48 % of 2-MIB and 0.77 % of geosmin were freely dissolved.

$$Binding\% = \frac{Ctotal - Cfree}{Ctotal} * 100 = \frac{ne(gel) - ne(fish)}{ne(gel)} * 100 \quad (3.1)$$

ne(gel) and *ne(fish)* mean the extraction amounts under equilibrium condition.

3.3.2.3 Same free concentration of analytes in binding matrices and gel model

Based on the binding percentage obtained in the last section, the same free concentration of 2-MIB and geosmin in the samples, with and without the presence of binding matrices, were prepared. Homogenized fish tissue containing 150 ng/g 2-MIB and geosmin and gel model containing 5.22 ng/g 2-MIB and 1.16 ng/g geosmin were prepared. Fig. 3.3 and 3.4 present the extraction time profiles of 2-MIB and geosmin in the systems with and without the presence of binding matrices containing the same free concentration of analytes. Fig. 3.5 and 3.6 compare the time constant profiles between gel model and fish tissue under this condition.

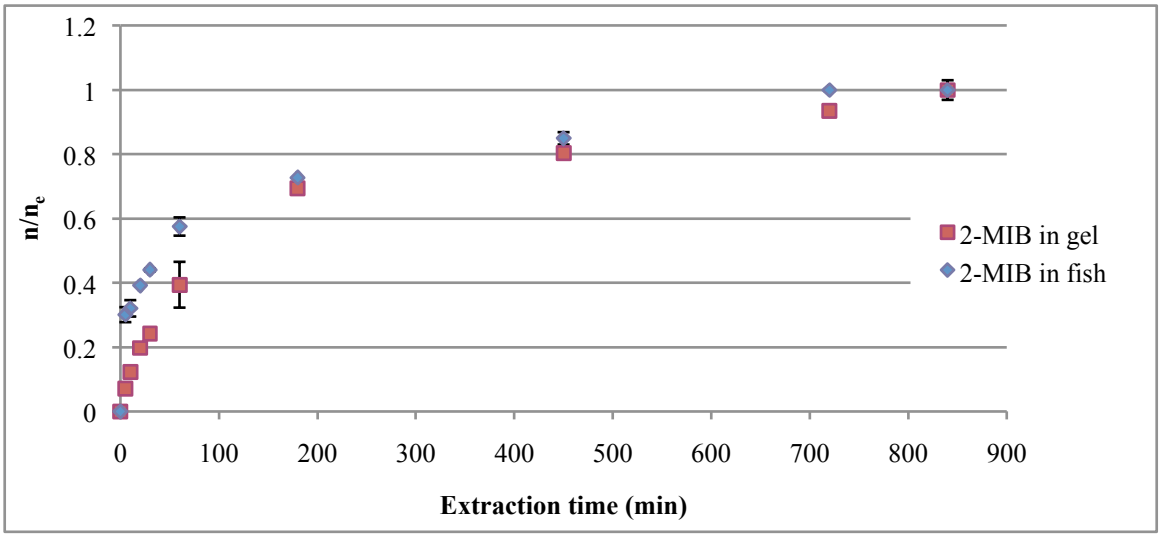


Figure 3.3 Extraction time profiles for the same free concentration of 2-MIB in gel and homogenized fish tissue

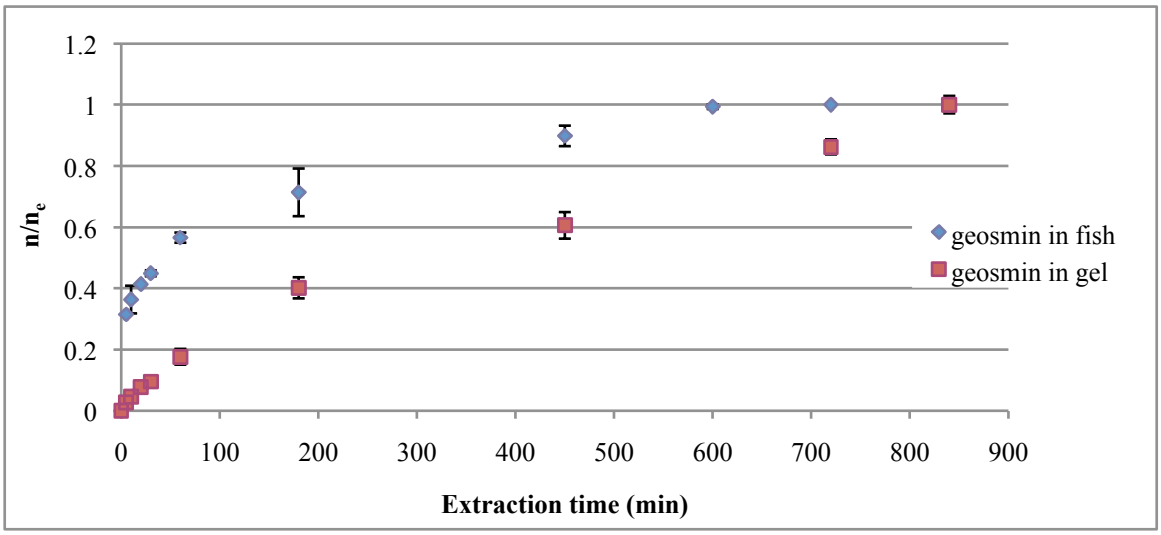


Figure 3.4 Extraction time profiles for the same free concentration of geosmin in gel and homogenized fish tissue

For geosmin, the equilibrium time for analytes with a binding matrix is shorter than that without a binding matrix. Therefore, as can be seen from the figures, the

kinetics of SPME extraction for geosmin can be affected by the presence of a binding matrix. For 2-MIB, the time constant in fish tissue was similar to that in gel, and the difference was within the standard deviation (Fig. 3.5); while for geosmin, the time constant in fish tissue was much greater than that in the gel. As a result, it can be concluded that binding indeed had an impact on the final equilibrium time in the way of affecting time constants. This faster equilibrium phenomenon can be explained by local depletion. The free dissolved analytes in the boundary layer around the fibre coating are depleted due to the extraction. With the presence of a binding matrix, analyte concentration in the boundary layer is compensated by dissociation from the analyte-binding matrix complex, which in turn increases the overall extraction speed and makes extraction of analytes in homogenized fish tissue reach equilibrium earlier than in the gel. The local depletion however was not a significant depletion compared to the total concentration of analytes in the sample. In reality, only 0.32 % of 2-MIB and 0.24 % of geosmin in total were consumed.

In addition, the time constants of both analytes in homogenized fish tissue in this experiment were the same as the one obtained from the previous experiments (section 2.3.5). However, to note, the time constants found in gel in the current experiment were smaller than the previous data. The explanation behind this comes from the fact that the free concentration in the gel of the previous experiment (1.0 μg/g for both analytes in section 3.3.2.1) was much higher than the current situation (5.22 ng/g for 2-MIB and 1.16 ng/g for geosmin). Therefore, the free-dissolved concentration was sufficient to be extracted by SPME fibres in previous experiments. In fish samples, the consistent time constants found were due to the fast dissociation of the analyte-binding complex, which

could compensate depleted free analytes immediately within the first 5-60 min. Oomen *et al.* concluded that the contribution of dissociated analytes to the uptake flux is expected only if (1) the rate-limiting step of the uptake process is diffusion through the boundary layer, (2) the concentration of the sorbed analyte is high, and (3) dissociation from the matrix is fast.[88] This would assist to understand the different shapes of time constant profiles of fish tissue and gel mentioned in section 3.3.2.1. In Fig. 2.10, the decreased slope of time constant profiles of both geosmin and 2-MIB in fish tissue indicated that after 60 min, the time constants of extraction and desorption decreased, which means the dissociation of analyte-binding matrix could not compensate depleted free contribution instantaneously. On the other hand, because there was no binding matrix in the gel system, the time constant kept the same all the process as long as the free concentration was not too low (such as 1.0 μg/g of geosmin and 2-MIB in the gel). Moreover, the value of the time constant in gel model under that condition was equal to that in the fish tissue.

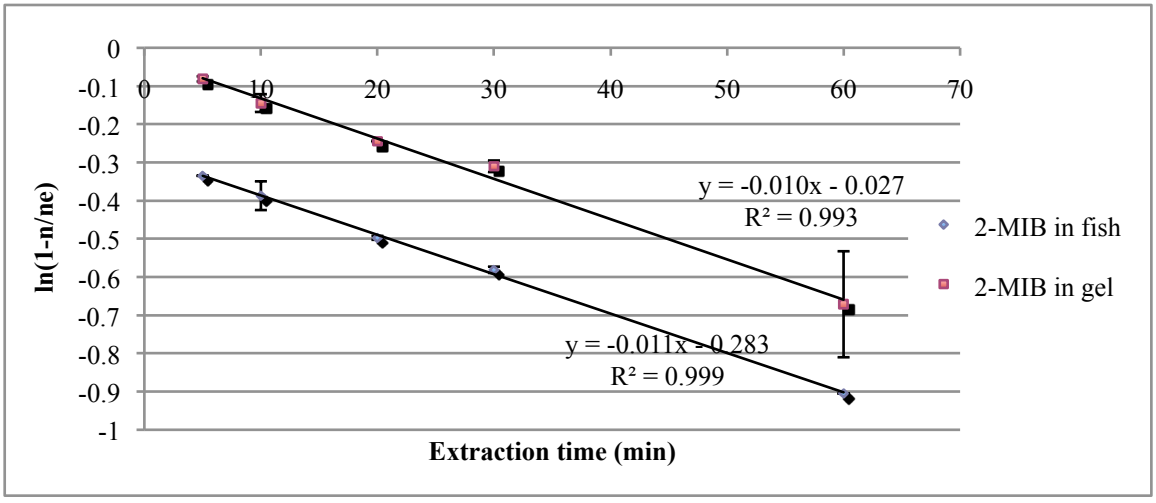


Figure 3.5 Time constant profiles for same free concentration of 2-MIB in gel and in homogenized fish tissue

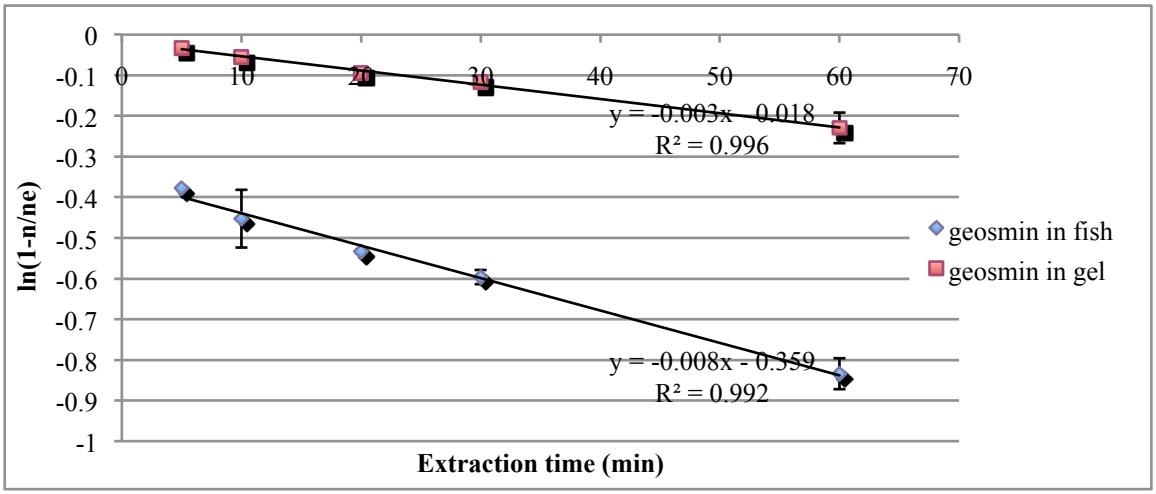


Figure 3.6 Time constant profiles for same free concentration of geosmin in gel and in homogenized fish tissue

3.3.2.4 Confirmation of consistent time constant under 30 min extraction

By using the on-fibre standardization calibration method, the same desorption and absorption time constants are inevitable. It has been proven that for fish tissue exhibiting a high contamination index (as in section 2.3.5 and section 3.3.2.1), the symmetry definitely exists. However, when low total concentration is present, gel system exhibits unsymmetrical results between desorption and absorption. For that reason, low concentration contaminated fish should always be inspected in order to verify the isotropy of kinetics.

Different concentrations of 2-MIB and geosmin in homogenized fish tissue were prepared and followed by a 30 min extraction. According to eq. 1.3, the ratio of total concentration for different samples should be equal to that of extraction amount under the same extraction conditions.

$$n = [1 - \exp(-at)]n_e = [1 - \exp(-at)]\frac{K_{fs}V_fV_s}{K_{fs} + V_s}C_0 \qquad (1.3)$$

Table 3.3 Total concentration effects on kinetics

Total con. (ng/g)	Extraction amount of 2-MIB (pg)	Extraction amount of geosmin (pg)
0.25	0.9±0.4	0.7±0.2
0.5	2.4±0.1	1.5±0.1
2.5	11.7±1.2	6.3±0.5
5.0	23.8±1.3	12.4±0.3
12.5	56.2±2.5	31.2±3.8
25.0	119.2±4.7	61.0±2.1

⋆each point 3 replicates, results followed by standard deviation.

In the current experiment, K_{fs}, V_f, and V_s were all equal for each absorption, and the ratio of each *n* showed to be similar to the ratio of each total concentration, which can be calculated by Table 3.3. Applied this results to eq. 1.3, it can be concluded that the extraction time constant for both analytes remained consistent in this concentration range. Desorption time constants of geosmin for homogenized fish tissue has already been proved to be equal to absorption time constant under the concentration of 25 ng/g (as shown in Fig. 2.9). Thus, it can be concluded that in the above concentration range, desorption and absorption process of geosmin followed sysmetry.

3.3.3 Verification of kinetic calibration method

3.3.3.1 Fibre constant measurement

Three different concentrations of homogenized fish tissue were prepared. Equilibrium extraction was performed to obtain the fibre constant (K_{fs}*V_f) value. As a result, 2-MIB and geosmin obtained the fibre constant 6.5 μL and 4.7 μL, respectively.

Table 3.4 Fibre constant determination

Homogenized fish tissue con. (ng/g)	2-MIB		Geosmin	
	Extraction amount (pg)	Fibre constant (μL)	Extraction amount (pg)	Fibre constant (μL)
5.0	32.0	6.4±0.4	22.7	4.5±0.4
10.0	65.3	6.5±0.3	48.4	4.8±0.6
50.0	330.8	6.6±0.6	238.6	4.8±0.2

⋆each point 3 replicates, results followed by standard deviation.

3.3.3.2 Verification of results using kinetic calibration method

To verify the on-fibre standardization calibration method, total concentrations of geosmin using the fibre constants obtained above were calculated. Table 3.5 shows the comparison of calculated and actual spiked concentrations. Because of the common ions present in 2-MIB and d_3-MIB, only d_3-geosmin was uploaded prior to the extraction, and only concentrations of geosmin were compared. The calculated concentrations are very similar to the actual concentrations.

Table 3.5 Comparison of calculated concentration and actual concentration

Spiked con. (ng/g)	Calculated con. (ng/g)
0.25	0.29±0.1
0.5	0.6±0.1
2.5	2.6±0.2
5.0	5.1±0.1
12.5	12.7±1.6
25.0	24.9±0.9

⋆each point 3 replicates, results followed by standard deviation.

3.4 Conclusion

In conclusion, 1 % gel (w/w) is the most suitable model to simulate fish tissue when taking into consideration of time constants. Binding indeed exists in the extraction process, but it does not affect the results of SPME measurment within the first 5-60 extraction time. Due to the fast dissociation rate of analyte-binding complex, free dissolved analytes could always be compensated by dissociated analytes. Therefore, the time constants stay consistent within the first 5-60 min extraction time, regardless of the change in concentration of binding matrices or total concentration of analytes in the sample. This discovery is very important for measurment using pre-determined rate calibration method (details in section 4.3.5.4), in which a constant sampling rate is required through the process of extraction. Lastly, the on-fibre standardization calibration method of geosmin was confirmed successfully by comparing calculated concentrations with actual spiked concentrations.

4 Chapter 4- *In-vivo* sampling of 2-MIB and geosmin in rainbow trout in RAS

4.1 Introduction

Off-flavor compounds determination has been investigated in many studies to date. Traditional sampling and sample preparation techniques have accomplished an accurate measuring of the concentration of analytes in fish, and obtaining very low detection limits.[34, 43-47] The drawbacks of such techniques are that they usually require extended time, large amount of solvent, complicated instrumentations, and most importantly, they are unsuitable in RAS reformation for tracking the dynamic process of bioaccumulation in living fish, which reflect the treatment of the outside living environment.

Recently, the simplicity and robustness of SPME technique has been applied to *in-vivo* determination of pharmaceuticals in fish.[59, 62,73] A significant advantage of SPME fibres is their ability to extract a variety of trace contaminants from fish tissue without lethal sampling. To date, only LC analysis was applied in determination of contamination in fish muscle, constraining the understanding of volatile compounds caused by microorganisms in water that have negative effects on fish either in the way of tasting or toxin.

According to the physiochemical properties of geosmin and 2-MIB, the relatively high log K_{ow} allows them to accumulate in fat tissue with higher amounts than in muscle. In this case, determination in fat tissue could be an optional sample position to reduce the sampling time or increase the extraction amount of analytes.

In this chapter, both muscle and fat tissue under the fish belly were sampled using kinetic calibration *in-vivo* SPME technique to determine the geosmin and 2-MIB amounts

in fish from RAS. The instrument parameters and calibration methods were optimized in order to compete with the human sensory threshold. Finally, the results were compared with microwave distillation-SPME (MD-SPME) method to validate the accuracy of *in-vivo* SPME.

4.2 Experimental section

4.2.1 Animals

All *in-vivo* fish experimental procedures were approved by the Animal Care Committee at University of Guelph (AUP No. 12R066). For this study, *in-vivo* sampling experiments were performed at Alma Aquaculture Research Station, which belongs to the University of Guelph. Fish tissue experimentation was approved by the local Animal Care Committee at University of Waterloo (AUP No. A-12-01). Rainbow trout (*Oncorhynchus mykiss*) were of marketable table size at 900$\pm$154 g (n=48).

4.2.2 Chemicals and materials

All geosmin, 2-MIB, their deuterated standards and solvents (methanol and acetone) were obtained in the same way as previous chapters. Chloroform for fat content measurement was purchased from EMD Chemicals Inc. (Damstadt, Germany).[59] SPME metal core fibres were purchased as previously. MS-222 (tricaine mesylate) used for anesthesia and fish diets were offered and implemented by Alma Aquaculture Research Station. The source of water in the station was from the river nearby, and was proved to

obtain off-flavor compounds under detection limit by using headspace SPME analysis technique.[93] The temperature of water was always kept at 8.5 °C.

4.2.3 *In-vivo* sampling system setup

Fish were acclimated with the diet in a flow-through system one week prior to experimentation. The system was located indoors and consisted of 16 replicates 0.729 m^3 square tanks. Each tank held four fish, three for experimental use and one for mortality. Before exposure to tainted water, the fish were deprived of food for 48 h. This was in order to minimize variations in metabolic rates and ventilation rates between individual fish with different feeding histories.[50] During exposure time, the fish were deprived of food as well.

The uptake of off-flavor compounds from contaminated water by fish as well as the *in-vivo* sampling experiments were both performed in a static, albeit oxygen offered system (no water flow rate). Due to the limited number of SPME fibres, only three round tanks were prepared for static system, and a total of nine fish were used each time. The volume of each round tank for the static system was 120 L, with 90 L of water inside, which was transferred from the flow-through system. The temperature of the water in the static tank was kept at 8.5 °C by settling each round tank into one flow-through tank.

4.2.4 Instrumentation

The instrumentation used was the Varian 4000 ion trap MS coupled with Varian 3800 GC. The GC parameters were the same as section 3.2.4. The scan modes of MS were optimized in this chapter for better limit of detection.

4.3 Results and discussion

4.3.1 Determination of pre-equilibrium extraction time

According to Zhou *et al.*'s conclusion on determining the pre-equilibrium absorption and desorption time, firstly to find the equilibrium time by absorption time profiles; and then to set 1/10 of the equilibrium time as the pre-equilibrium time.[56] The reason for choosing this time is, for most of the cases, this point is close to the crossing point of absorption and desorption time profiles. That half of the amount equilibrium extraction from sample matrices as well as a certain amount of desorption from the preloading fibre can be observed at this time point. Fig. 4.1 illustrates the crossing time point for geosmin is about 30 min, which is similar to 2-MIB, shown in Fig. 2.9. Although this pre-equilibrium time was not at 1/10 of equilibrium time, which should be around 70-80 min, the extraction amount and desorption amount were both found to be acceptable. Moreover, the 30 min pre-equilibrium time is more time-efficient, and, most importantly, it is within the first 5-60 min of the extraction process, during which the time constants were proved to be consistent.

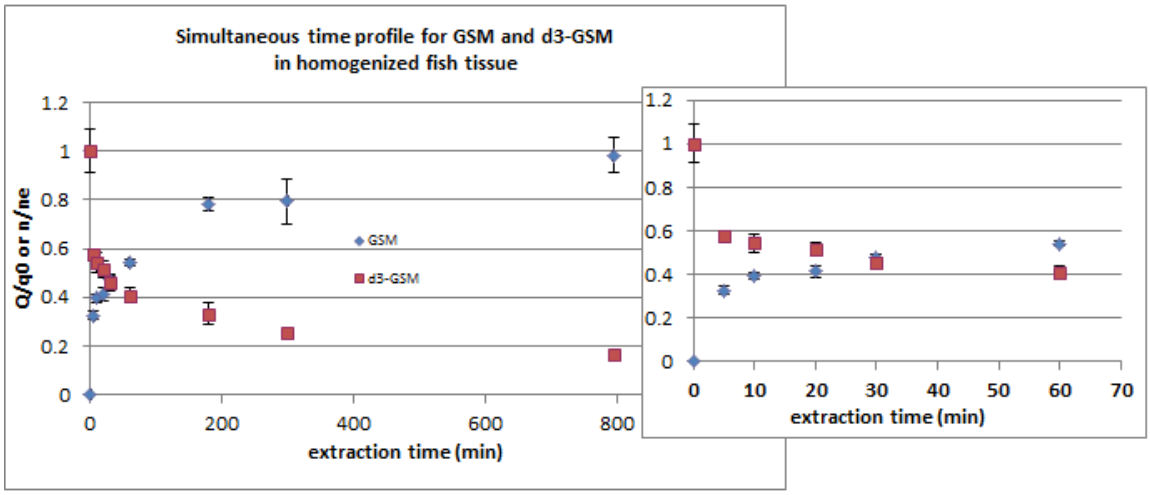


Figure 4.1 Absorption and desorption time profiles for geosmin; the enhanced part shows the crossing point of the two profiles.

4.3.2 Mass spectrometry scan mode optimization

Due to the common ions shared by d_3-MIB and 2-MIB, the ions which could have been selected to represent these two analytes have insufficient intensity. The detection limit using on-fibre standardization method to determine 2-MIB was 10.0 ng/g, which was more than 10 times of the human sensory threshold. On the contrary, geosmin could be isolated from its deuterated form easily by using base ions. Therefore, only geosmin performed on-fibre standardization calibration method, and 2-MIB would use the pre-determined sampling rates described in the end of section 1.2.3.2. In that case, *m/z* 95, the base ion of 2-MIB would be utilized to optimize the instrumentation analysis.

In order to achieve a low detection limit, a proper MS method should be developed and optimized. In the current experiment, three scan modes were compared. A 0.5 µL of 2-MIB and geosmin methanol solution with different concentrations was spiked

on the SPME fibre to introduce analytes, and the RSD for uploading procedure was 2.1 %. The ionization technique used was internal EI mode.

Full scan mode Quantification ions *m/z* 95 and 112 were used for 2-MIB and geosmin, respectively. Scan range was *m/z* 50-200. Emission current was set at 10 μAmps. Limit of detection of full scan was 0.5 pg for geosmin and 2.5 pg for 2-MIB, respectively.

MS/MS mode Another feature of the ion trap detector is MS/MS mode, which can improve the sensitivity of detection, especially for complex matrices. Fig. 4.2 shows the main fragment origins of geosmin. The geosmin molecule after isolation of ion *m/z* 112 and subsequently, the collision-induced dissociation (CID) yielded abundant ions *m/z* 97 and *m/z* 83.

In the ion trap, the energy of precursor ions can be increased either by non-resonant or resonant excitation method. According to the results found in the current experiment, both methods would work equally well; the CID spectra were shown to be very similar. Based on the suggestions of the Varian 4000 GC/MS Software Operation Manual, if the objective is to achieve maximum signal-to-noise (S/N) for a product ion, resonant CID does tend to concentrate fragmentation into fewer product ions. Therefore, the resonant CID was utilized for both analytes.

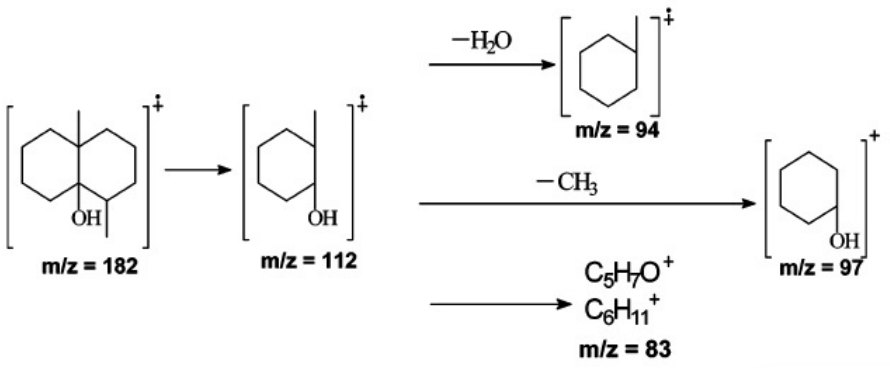


Figure 4.2 Main fragments origins of geosmin[94]

Table 4.1 MS mode parameters for 2-MIB and geosmin

Parameters	Geosmin	2-MIB
Precursor ion (*m/z*)	95	112
Waveform type	Resonant	Resonant
Excitation storage level (*m/z*)	69.3	47.9
Excitation amplitude (volt)	1.0	1.0
Product ion start mass (*m/z*)	80	66
Product ion end mass (*m/z*)	100	92
Quantification ions (*m/z*)	97, 83	67, 91
Emission current (μAmps)	80	80
Maximum ionization time (μsec)	65,000	65,000

There are three approaches in internal EI mode to increase the ion signal: (1) raise the emission current, (2) raise the multiplier voltage, and (3) increase the maximum ionization time.[95] The range of emission current for internal EI mode is 10-100 μAmps. Because higher emission currents decrease the life of the filament, the emission current

was not set at its highest level, with 80 μAmps as the standard. Maximum ion time was increased to 65,000 μsec, which is the largest available setting. Table 4.1 shows the parameters of MS/MS mode for 2-MIB and geosmin. The detection limit of MS/MS mode for both analytes was 0.5 pg.

Selected Ion Storage (SIS) mode Same as in MS/MS mode, in order to increase the sensitivity, emission current (80 μAmps) and maximum ionization time (65,000 μsec) were set at a high level. Ions *m/z* 112, 126 and 182 were selected for geosmin, and ions *m/z* 95, 107 and 168 for 2-MIB. The detection limit of SIS mode for standard spiking on the fibre was 0.25 pg for geosmin, and 0.5 pg for 2-MIB.

Lastly, the comparison of S/N for these three modes was made by analyzing 0.5 μL of 10 ng/mL geosmin and 2-MIB methanol solution spiked on fibre. Table 4.2 shows that MS/MS and SIS mode achieved the highest S/N for 2-MIB and geosmin, respectively.

Table 4.2 Three scan modes S/N comparison

	Full scan	SIS	MS/MS
2-MIB	33	37	58
Geosmin	45	236	146

4.3.3 Limit of detection (LOD)

Description of the limit of detection measurement followed the EPA procedure for the determination of Method Detection Limit (MLD) was as follows:[96]

1) Made an estimate of the detection limit by making a homogenized fish tissue sample with the concentration value that corresponds to Varian 4000 S/N, which was 5. (2-MIB: 0.25 ng/g; geosmin: 0.1 ng/g)

2) Prepared homogenized fish tissue that was free of analyte.

3) Prepared another same sample as above, containing 0.1 ng/g geosmin and 0.25 ng/g 2-MIB. The measured level of the 2-MIB and geosmin were 1.45 and 1.85 times of the estimated detection limit.

4) Repeated step 3) for eight continuous days.

5) The standard deviation of the replicate measurements was:

s(2-MIB)= 0.07 ng/g

s(geosmin)= 0.04 ng/g

6) Computed the MDL:

MDL (2-MIB$)=t_{n-1,1-\alpha=0.99}*s$= 2.998*0.07=0.21 ng/g

MDL (geosmin)$=t_{n-1,1-\alpha=0.99}*s$= 2.998*0.04=0.12 ng/g

Although the detection limits of both analytes using direct immersion SPME in homogenized fish tissue were not as low as other methods,[46, 47] it is still 4.5 times lower for 2-MIB and 8 times lower for geosmin than the human sensory threshold (section 1.2.2). There is no regulation about the relationship between detection limit and threshold for off-flavor compounds in food, but FDA stresses that the detection limit of an analytical method should be no more than its threshold. In Jones *et al.*'s report, for dredged sediment disposal in ocean or island water, the limit of detection should be 3-5 times lower than its threshold.[97] However, as described before, geosmin and 2-MIB do

not have any negative health effects on human beings Therefore, the detection limit obtained in the current experiment should be satisfactory.

4.3.4 Fibre repeatability and reproducibility

Commercial fibres have the advantages of good repeatability for intra-fibre and good reproducibility for inter-fibre sampling. Therefore, data normalization is not necessary due to the variance of fibres. A comparison was performed using nine used fibres (each one has been used for extraction from fish tissue for at least 20 times) to extract from the headspace of the deuterated standards generator vial. Fig. 4.3 displays the good repeatablility and reproducibility of the nine commercial fibres used in this experiment. Duplicates were operated for each fibre, and the RSDs for both compounds were less than 4.7 %. Variance caused by different fibres was within the standard deviation.

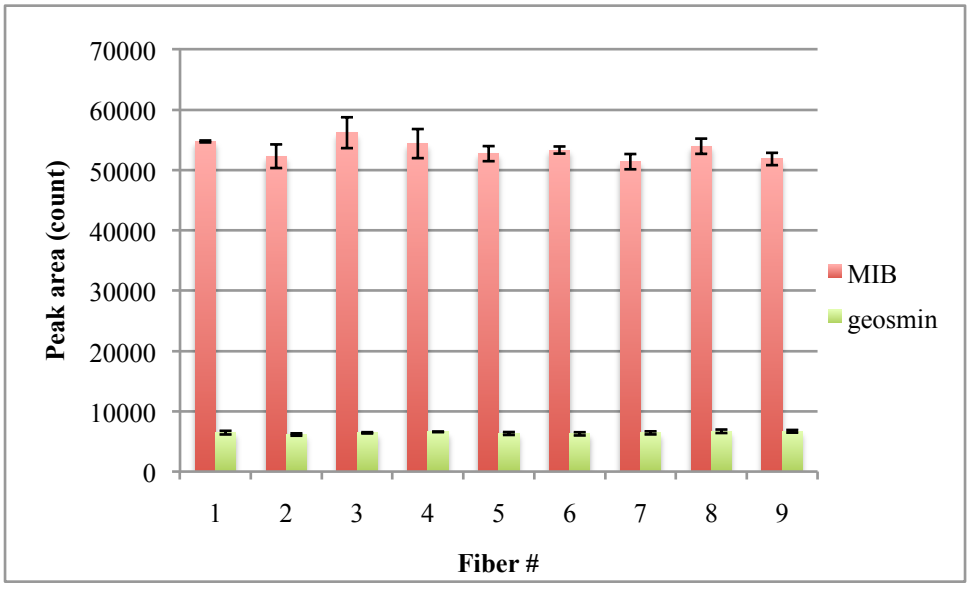


Figure 4.3 Nine commercial fibres reproducibility and repeatability comparison

4.3.5 Real sample application

4.3.5.1 Preliminary uptake experiment

An experiment using *in-vivo* sampling was performed by spiking geosmin and 2-MIB methanol solution into a static system prior to exposing fish inside contaminated water. The concentration of off-flavor compounds in water was decided based on Robertson *et al.*'s study,[50] and 5 mL of 9 μg/mL geosmin and 2-MIB standards in methanol were first spiked to 90 L of water to make an initial concentration of 0.5 ng/mL. The uptake experiment was run for 24 h with fish samples (n=9) in three tanks. After 24 h, *in-vivo* sampling SPME sampling was performed on each fish for 30 min, and fibres were brought back to the lab for instrumental analysis. Obvious peak intensity was observed for all seven collected fibres, with two fibres being lost as they fell down in the water due to the struggling of fish.

This leads to the conclusion that a lower concentration of analytes in water (lower than 0.5 ng/ml) could be used. In addition, prior to sampling, three fish were used as control by spiking 5 mL methanol into 90 L water, and, after 24 h exposure, no off-flavor compound was detected.

4.3.5.2 Operation procedure

As mentioned above, fibre fastness inside fish muscle during the sampling process was an issue. The previous inserting position was directly under dorsal fin, however, as the fish recovered from anesthesia, the movement of body muscle changed the fibre inserting position, even as far as removing the fibre. Thus, after considering the

swimming movement of fish, the final inserting position was relocated to the front part muscle of the body. (as shown in Fig. 4.4) By changing location of sampling, fibres stayed in place in subsequent experiments.

Figure 4.4 SPME fibre inserting position for *in-vivo* sampling in fish muscle

The procedure for operating *in-vivo* sampling is as follows: (1) fibres were prepared by uploading deuterated geosmin standard from headspace of pump oil solution; (2) before each SPME sampling, fish were anesthetized in a 80 µg/mL MS-222 water solution for the duration of approximately 1 min; (3) an 18 gauge needle was used to pierce the fish (not as long as SPME fibre), and a 21 gauge long stainless steel wire was introduced into the same perforation and penetrated to the same length as SPME fibre; (4) SPME fibre was inserted, and the probe was pushed all the way to the end; (5) following fibre placement, fish were placed back in the static tank; (6) after 30 min SPME extraction, the fish was anesthetized again, (7) the fibre was removed, gently washed with

nanopure water, wiped with Kimwipe, sealed with teflon cap, wrapped with aluminum foil, and stored in the dry ice until GC/MS analysis.

4.3.5.3 Real sample isotropy verification

Symmetry characteristic of absorption and desorption of both geosmin and 2-MIB under *ex-vivo* conditions using fish tissue has been proven. (section 2.3.5) Isotropy testing using live fish is discussed in this section, with nine fish from three tanks used as samples. Due the limited number of fibres, only three time points (10, 20 and 30 min) were performed. In order to separate deuterated 2-MIB and 2-MIB, a high concentration of analytes (5.0 ng/mL) in water was prepared. After exposure for 24 h in contaminated water, three fish were picked out from three tanks respectively as one time point. Therefore, each time point has three replicates, all from three different tanks. Table 4.3 illustrates the isotropy results obtained by using live fish. Compared to the time constants obtained with homogenized fish tissue and non-homogenized fish tissue, *in-vivo* sampling SPME also had similar rate constants, and most importantly, the desorption and absorption processes displayed isotropy.

Table 4.3 Isotropy of geosmin and 2-MIB in live fish using *in-vivo* SPME sampling

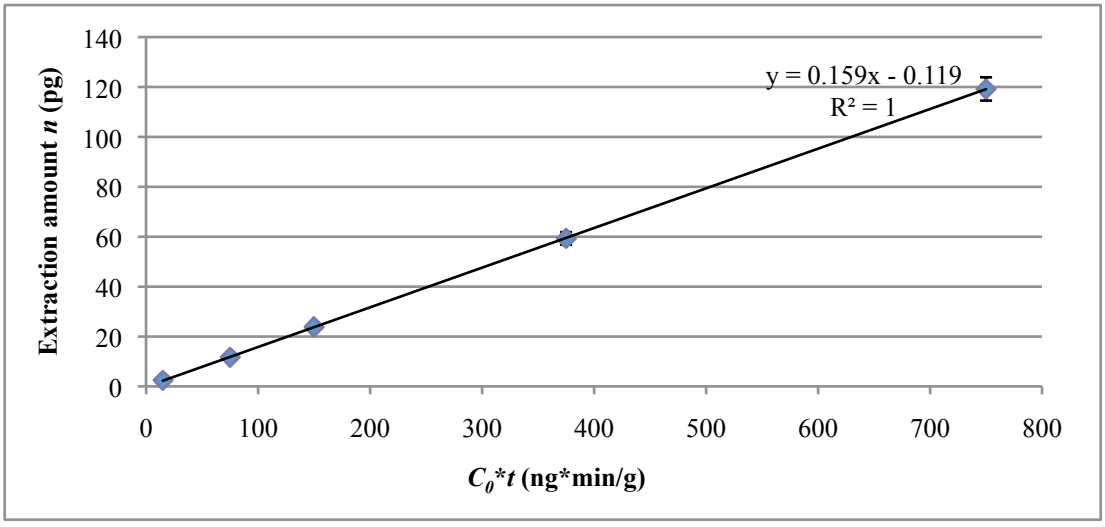
	Desorption rate (/min)	Absorption rate (/min)
2-MIB	0.010±0.003	0.013±0.006
Geosmin	0.012±0.003	0.011±0.007

4.3.5.4 Using pre-determined sampling rates to determine concentration of 2-MIB in fish

A brief introduction of this kinetic calibration method was presented in section 1.2.3.2. Within a linear model, it is assumed that the rate of mass transfer or sampling rate remains constant throughout the duration of sampling. The relationship between the concentration of target analytes (C_0) in the sample matrices and the extracted amount of analytes (n) at time t can be expressed using eq.1.7:

$$C_0=n/R_st \quad (1.7)$$

It has been already demonstrated previously in this study that within the first 5-60 min of extraction time, the time constant of 2-MIB was 0.012 /min. Moreover, according to the SPME uptake profile, the kinetics of absorption stays linear before t_{50}, which is about 30 min for 2-MIB. Therefore, the determination of concentrations of 2-MIB in fish is quantified using this method.



⋆each point 3 replicates.

Figure 4.5 Determination of R_s of 2-MIB in homogenized fish tissue

Different concentrations (0.5, 2.5, 5.0, 12.5, 25.0 ng/g) of 2-MIB in homogenized fish tissue were prepared to obtain the extraction amount *n* under 30 min sampling time, and all the other conditions were performed the same as *in-vivo* sampling SPME. Ploting C_0**t vs. n*, the slope obtained was R_s. (as shown in Fig. 4.5). As a result, the R_s value was determined to be 1.6E-04 g/min. Since 30 min was always set as the sampling time, and to simplify data processing, $R_s t$ was calculated as 0.0048 g.

4.3.5.5 Low contamination *in-vivo* sampling experiment results

Nine fish were exposed to low concentration of geosmin and 2-MIB (0.05 ng/mL) in water. The concentration of preloaded d_3-geosmin in pump oil was 50 μg/g, and the headspace extraction time was 10 s. On-fiber standardization calibration method was applied for the quantification of geosmin, and 2-MIB used pre-determined sampling rate approach. Results are shown along with fish weight, sex, fat content and analyte concentration in Table 4.4. Fat content of rainbow trout was assessed according to Zhang *et al.*'s method.[73] With a three-fish-per-tank limit, fish No. 1, 2, and 3 were in the same tank, followed by the other six fish, with the total tank amount of three. According to the results, there is no obvious relationship between mass, fat and concentration of analytes. Fish from different tanks do not exhibit a significant difference. To confirm the accuracy of results, a traditional method was used for validation in section 4.3.6.

Table 4.4 Low concentration of off-flavor compounds in fish

Fish No.	Mass (g)	Sex	Fat content (%)	2-MIB (ng/g)	Geosmin (ng/g)
1	1013.0	F	3.4	3.4	2.6
2	883.3	M	3.0	3.9	3.0
3	855.4	M	2.7	2.8	2.1
4	1003.2	M	3.7	2.6	2.0
5	894.1	M	1.6	3.3	3.4
6	985.6	M	2.1	3.4	2.2
7	772.6	M	2.9	4.9	6.1
8	958.3	M	2.7	3.8	3.0
9	896.0	M	3.7	4.5	5.2

4.3.6 Validation of *in-vivo* SPME sampling using microwave distillation-SPME

Microwave distillation sample preparation was done by one of the collaborators in Laval University, and the distillates were sent back to Waterloo for instrumental analysis. Microwave distillation and SPME headspace extraction procedures were executed in accordance with Lloyd *et al.*'s publication.[46] Same instrument parameters were used as *in-vivo* sampling analysis of fish. External calibration method was utilized for quantification by spiking known amount of standards into nanopure water in order to prepare different concentrations of water samples. The recovery of the microwave distillation step reported by Laval University was 6.1 % for geosmin. Although they did not supply recovery information about of 2-MIB, such information was found in previous research,[8, 46, 50] The recoveries for geosmin and 2-MIB using microwave distillation methods and extracting from fish were almost the same. Therefore, we assumed that

microwave recoveries for 2-MIB and geosmin were both 6.1 %. Table 4.5 displays the results of off-flavor compounds concentrations of nine low contaminated fish using two sample preparation methods. The comparison states that there is no significant difference of measurement results between *in-vivo* SPME and MD-SPME, which validates the use of *in-vivo* sampling SPME technique.

Table 4.5 Results comparison between MD-SPME and *in-vivo* SPME

	2-MIB in fish (ng/g)		Geosmin in fish (ng/g)	
Fish No.	*In-vivo* SPME	MD-SPME	*In-vivo* SPME	MD-SPME
1	3.4	3.0	2.6	3.0
2	3.9	3.9	3.0	3.7
3	2.8	3.6	2.1	2.9
4	2.6	4.4	2.0	3.0
5	3.3	3.3	3.4	4.6
6	3.4	4.6	2.2	3.7
7	4.9	3.7	6.1	5.9
8	3.8	5.4	3.0	5.1
9	4.5	4.4	5.2	5.0

4.3.7 An optional sampling position by using fat-specific tissue in fish

As mentioned in section 1.1.3, fat is a part of fish tissue that accumulates more geosmin and 2-MIB than muscle, considering the log K_{ow} of these two analytes. Unfortunately, at this time *in-vivo* sampling by inserting SPME fibre into fat under belly was not accomplished, due to the severe struggle of fish. One possible solution to fasten

the fibre under the belly during the *in-vivo* extraction process is to use tape facilitate the fibres to remain in place after the fish recovers. However, due to time limitations, SPME extraction in fat-specific tissue in fish was performed *ex-vivo* instead of *in-vivo*. A 10 min analyte extraction from fat was compared with a 30 min extraction from muscle using three contaminated fish, with each fish being sampled two times (each side one time). Results are shown in Table 4.6.

Table 4.6 Fat extraction *vs.* muscle extraction

Fish number	2-MIB		Geosmin	
	10 min in fat (pg)	30 min in muscle (pg)	10 min in fat (pg)	30 min in muscle (pg)
1 (replicate1/2)	5.7/6.5	6.1/5.9	2.6/2.5	2.6/2.4
2 (replicate1/2)	8.3/8.0	6.2/7.2	3.9/5.0	2.4/4.2
3 (replicate1/2)	7.2/6.7	6.9/6.3	3.2/2.9	3.0/2.9

According to the data, a 10 min extraction in fat obtained a similar amount of analytes to a 30 min extraction in muscle. Although fat is usually not marketable, it still can be an optional sampling position to measure the concentration of geosmin and 2-MIB in fish, with sampling time being decreased by 2/3.

4.4 Conclusion

In-vivo SPME sampling was validated by traditional method MD-SPME. On-fibre standardization calibration and pre-determined extraction rate methods were used for

quantification of geosmin and 2-MIB concentration in fish muscle, respectively. MS/MS was chosen as a sensitive MS scan mode for 2-MIB instrumental analysis, and SIS mode was effective for geosmin analysis. Detection limits by using *in-vivo* SPME technique were 0.21 ng/g and 0.12 ng/g for 2-MIB and geosmin, respectively, which were much lower than human sensory threshold. Isotropy under *in-vivo* conditions was confirmed by spiking a large amount of off-flavor compounds in water, and time constants were similar to those obtained under *ex-vivo* conditions. At last, an optional sampling position was suggested to extract geosmin and 2-MIB from fat tissue in fish, which could be more

5 Chapter 5- Summary

An *in-vivo* SPME sampling technique used to measure the concentration of off-flavor compounds in fish was developed. Compared with traditional methods, it has the advantages of time efficiency, simplicity of procedure, and most important, the capability of monitoring target compound concentration changes when sampling from the same individual fish.

Kinetic calibration methods were used for quantification. On-fibre standardization method was used to measure geosmin, while pre-determined kinetic rates were employed to determine the concentration of 2-MIB in live fish. Due to instrumental limitations, the on-fibre standardization calibration method could not be performed on both off-flavor compounds being measured, however, the isotropy character of desorption of internal standards from fibre and absorption of analytes onto fibre for these two compounds was confirmed. The time constants for 2-MIB and geosmin in the first 5-60 min extraction time were 0.0012 /min and 0.008 /min, respectively, and the values did not show a significant difference among homogenized fish tissue, non-homogenized fish tissue, and live fish. The method was sensitive enough to detect off-flavor compounds with a LOD of 0.21 ng/g for 2-MIB and 0.12 ng/g for geosmin, both which were far below the reported human sensory threshold of 0.9 ng/g for both compounds. Moreover, in addition to the sampling position in marketable muscle tissue, an optional inserting position in the lipid under belly could be an effective approach to save 2/3 of total sampling time.

Binding was proven to exist in the extraction process of 2-MIB and geosmin; however, due to the fast dissociation of the analytes-binding matrices, binding did not

have a significant effect on the absorption or desorption rates of these two analytes at 30 min pre-equilibrium extraction time.

In conclusion, this research indicates that *in-vivo* SPME technique could effectively monitor the concentration of geosmin and 2-MIB in fish.

References

1. H. Rosenthal, J.D. Castell, K. Chiba, J.R.M. Forster, V. Hilge, H. Hogendoorn, R.D. Mayo, J.F. Muir, K.R. Murray, J. Petit, G.A. Wedemeyer, F. Wheaton, J. Wickins,. (1986). *EIFAC , 100*.

2. C.I.M. Martinsa, E.H. Eding, M.C.J. Verdegem, L.T.N. Heinsbroek, O. Schneider, J.P. Blancheton, E. Roque d'Orbcastel, J.A.J. Verreth. (2010). *Aquacult. Eng. , 43*, 83-93.

3. M.C.J. Verdegem, R.H. Bosma, J.A.J. Verreth,. (2006). *Int. J. Water Resour. Dev. , 22*, 101-113.

4. R.H. Piedrahita. (2003). *Aquaculture , 226*, 35-44.

5. S.T. Summerfelt, M.J. Sharrer, S.M. Tsukuda, M. Gearheart,. (2009). *Aquaculture , 40*, 17-27.

6. Y. Zohar, Y. Tal, H.J. Schreier, C. Steven, J. Stubblefield, A. Place,. (2005). *Urban Aquaculture* , 159-171.

7. B. Zaitlin, S. B. Watson. (2006). *Water Res. , 40*, 1741-1753.

8. C.C. Grimm, S.W. Lloyd, R. Batista, P.V. Zimba. (2000). *J. Chromatogr. Sci. , 38*, 289-296.

9. N.N. GERBEK. (1969). *J. Antibiot. , 22*, 508-509.

10. A.A. Rosen, C.I. Mashni, R.S. Safferman. (1970). *Water Treat. Exam. , 19*, 106-119.

11. M. Yurkowski, J.L. Tabachek. (1976). *J. Fish. Res. Board Can. , 33*, 25-35.

12. M. Yurkowski, J.L. Tabachek. (1974). *J. Fish. Res. Board Can. , 31*, 1851-1858.

13. P.E. Persson, R. K. York. (1978). *Aqua Fenn. , 8*, 89-90.

14. F. Juttner, S. B. Watson. (2007). *Appl. Environ. Microbiol , 73*, 4395-4406.

15. J.A. Maga. (1987). *Food Rev.Int. , 3*, 269-284.

16. L.D. Tyler, T.E. Acree, R.M. Butts,. (1978). *J. Agr. Food Chem. , 26*, 1415-1417.

17. E.H.Polak, J.Provasi. (1992). *Chem. Sense , 17*, 23-26.

18. P.E. Persson, R. K. York. (1978). *Aqua Fenn. , 8*, 83.

19. Chemspider. (2011, April 17). *ChemSpider | geosmin | C12H22O*. Retrieved December 15, 2009, from Database of Chemical Structures and Property Predictions: http://www.chemspider.com/Chemical-Structure.27642.html

20. Chemspider. (2011, April 17). *ChemSpider | 2-Methylisoborneol | C11H20O*. Retrieved December 15, 2009, from Database of Chemical Structures and Property Predictions: http://www.chemspider.com/Chemical-Structure.20474023.html

21. P. Howgate. (2004). *Aquaculture , 234*, 155-181.

22. Q. Hu, M. Sommerfeld, L. Baker, P. Westerhoff,. (2003). *J. Water. Supply Res. Technol. – Aqua , 52*, 545-554.

23. G. Izaguirre, W.D. Taylor,. (1998). *Water Res. , 32*, 1673-1677.

24. C. Klausen, M.H. Nicolaisen, B.W. Strobel, F. Warnecke, J.L. Nielsen, N.O.G. Jorgensen. (2005). *FEMS Microbiol. Ecol. , 52*, 265-278.

25. E. Lanciotti, C. Santini, E. Lupi, D. Burrini,. (2003). *J. Water. Supply Res. Technol. – Aqua , 52*, 489-500.

26. R. Srinivasan, G.A. Sorial. (2011). *J. Environ. Sci. , 23*, 1-13.

27. C.M. Fetterolf. (1964). *Engng Bull. Purdue Unir., Engng Ext. Ser. , 48*, 174-182.

28. K.E. Clark, A.P.C. Gobas, D. Mackay,. (1990). *Environ. Sci. Technol. , 24*, 1203-1213.

29. I. From, V. HorByck. (1984). *Can. J. Fish. Aquat. Sci. , 41*, 1224-1226.

30. J. Boetius. (1954). *Meddr Danm. Fisk.-og Havunders. , 1*, 1-8.

31. D.L. Shumway, J. R. Palensky,. (n.d.). *Environmental Protection Agency, Ecology Research Series EPA-R3-73- 010.*

32. L.L. Maligalig, J.F.Caul, R. Bassette, O.W. Tiemeier,. (1975). *J. Fd Sci. , 40*, 1242-1245.

33. P.B. Johnsen, S.W. Lloyd,. (1992). *Cmo. J. Fish. Aquar. Sci. , 49*, 2406-2411.

34. P.B. Johnsen, S.W. Lloyd, B.T. Vinyard, C.P. Dionigi,. (1996). *J. World. Aquacult. Soc. , 27*, 15-20.

35. W.B. Neely. (1979). *Environ. Sci. Technol. , 13*, 1506-1510.

36. J.W. Nichols, J.M. McKim, G.J. Lien, A.D. Hoffmann, S.L. Bertelsen, C.A. Gallinat,. (1993). *Aquat. Toxicol. , 27*, 83-112.

37. G. Ouyang, D. Vuckovic, J. Pawliszyn. (2011). *Chem. Rev. , 111*, 2784-2814.

38. J. Pawliszyn. (2003). *Aust. J. Chem. , 56*, 155-158.

39. J. Pawliszyn. (2003). *Anal. Chem. , 75*, 2543-2558.

40. C.L.Arthur, J. Pawliszyn,. (1990). *Anal. Chem. , 62*, 2145-2148.

41. R.T. Kennedy, J.E. Thompson, T.W. Vickroy,. (2002). *J Neurosci. Methods , 114*, 39-49.

42. M.J. Cano-Cebrian, T. Zornoza, T. Polache, L. Granero,. (2005). *Curr. Drug Metab. , 6*, 83-90.

43. M.J. McGuire, S.W. Krasner, C.J. Hwang, G.Izaguirre,. (1981). *J. Am. Water Works Assoc. , 73*, 530-537.

44. J.F. Martin, C.P. McCoy, W. Greenleaf, L. Bennett. (1987). *Can. J . Fish. Aqucmm. Sci. , 44*, 909-912.

45. E.D. Conte, C. Shen, D.W. Miller. (1996). *Anal. Chem. , 68*, 2713-2716.

46. S.W. Lloyd, C.C. Grimm. (1999). *J. Agric. Food Chem. , 47*, 164-169.

47. M.A. Petersen, G. Hyldig, B.W. Strobel, N.H. Henriksen,N.O. G. Jørgensen. (2011). *J. Agric. Food Chem , 59*, 12561-12568.

48. P. E. Persson. (1980). *Water Res. , 14*, 1113-1118.

49. R.T. Lovell, I.Y. Lelana, C.E. Boyd, M.S. Armstrong,. (1986). *T. Am. Fish Soc. , 115*, 485-489.

50. R.F. Robertsona, K. Jaunceyb, M.C.M. Beveridgec, L.A. Lawton. (2005). *Aquaculture , 245*, 89-99.

51. P.E. Persson. (1980). *J. Agr. Food Chem. , 28*, 1344-1345.

52. I.Y. Lelana. (1987). *Geosmin and off-flavor in channel catfish.* PhD thesis., Auburn University, AL, USA.

53. G. Ouyang, J. Pawliszyn,. (2007). *J. Chromatogr. A , 1168*, 226-235.

54. J. Pawliszyn. (2009). *Handbook of Solid Phase Microextraction.* Beijing: Chemical Industry Press.

55. J. Ai. (1997). *Anal. Chem. , 69*, 1230-1236.

56. S. Zhou, W. Zhao, J. Pawliszyn. (2008). *Anal. Chem. , 80*, 481-490.

57. Y. Chen, J. Pawliszyn. (2004). *Anal. Chem. , 76*, 5807-5815.

58. G. Ouyang, S. Cui, Z. Qin, J. Pawliszyn,. (2009). *Anal. Chem. , 81*, 5629-5636.

59. G. Ouyang, K.D. Oakes, L. Bragg, S. Wang, H. Liu, S. Cui, M.R. Servos, D.G. Dixon, J. Pawliszyn. (2011). *Environ. Sci. Technol. , 45*, 7792-7798.

60. S. Risticevic, H. Lord, T. Górecki, C.L Arthur, J. Pawliszyn. (2012). *Nature Protocols , 5*, 122-139.

61. (2012). Retrieved from Canada (English)| Sigma-Aldrich: http://www.sigmaaldrich.com/canada-english.html

62. S. Zhou, K.D. Oakes, M.R. Servos, J. Pawliszyn. (2008). *Environ. Sci. Technol. , 42*, 6073-6079.

63. A. Jahnkea, P. Mayer. (2010). *J. Chromatogr. A , 1217*, 4765-4770.

64. H.J.M. Verhaar, F.J.M. Busser, J.L.M.Hermens,. (1995). *Environ. Sci. Technol. , 29*, 726-734.

65. J.T. Wu, F. J. (1988). *Arch. Microbiol. , 150*, 580-583.

66. W. Zhao, G. Ouyang, J. Pawliszyn. (2007). *Analyst , 132*, 256-261.

67. S. Ulrich. (2000). *J. Chromatogr. , 902*, 167-194.

68, M.B. Heringa, J.L.M. Hermens,. (2003). *TrAC-Trends Anal. Chem. , 22*, 575-587.

69. K.F. Poon, P.K.S. Lam, M.H.W. Lam,. (1999). *Chemosphere , 39*, 905-912.

70. K.F. Poon, P.K.S. Lam, M.H.W. Lam,. (1999). *Anal. Chim. Acta , 396*, 303-308.

71. M. Polo, V. Casas, M. Llompart, C. Gacı´a-Jares, R. Cela,. (2006). *J. Chromatogr. A , 1124*, 121-129.

72. S. Wang, K.D. Oakes, L.M. Bragg, J. Pawliszyn, G. Dixon, M.R. Servos. (2011). *Chemosphere , 85*, 1472-1480.

73. X. Zhang, K.D. Oakes, S. Cui, L. Bragg, M. R. Servos, J. Pawliszyn. (2010). *Environ. Sci. Technol. , 44*, 3417-3422.

74. (2012, 5 27). *Triton X-100 review*. Retrieved from http://www.labome.com/review/Triton-X-100.html

75. P.T Salo, J.A Beye, R.A Seerattan, C.A Leonard, T.J Ivie, R.C Bray. (2008). *Can. J. Surg. , 51*, 167-172.

76. R. McCallum, P. Pendleton, R. Schumann, M.-U. Trinh. (1998). *Analyst , 123*, 2155-2160.

77. J-P.F.P. Palmentier, V.Y. Taguchi, S.W.D. Jenkins, D.T. Wang, K-P. Ngo, D.Robinson. (1998). *Water Res. , 32*, 287-294.

78. F.D. Gunstone, J.L. Harwood, F.B. Padley. (2007). *The Lipid Handbook.* CRC Press.

79. J.D. Rabinowitz. (2011). *Anal. Chem. , 83*, 9114-9122.

80. B.O. Keller, J. Suj, A.B. Young, R.M. Whittal. (2008). *Anal. Chim. Acta , 627*, 71-81.

81. J. Poerschmann, F.-D. Kopinke, J. Pawliszyn,. (1997). *Environ. Sci. Technol. , 31*, 3629-3636.

82. W.H.J. Vaes, E. Urrestarazu Ramos, H.J.M. Verhaar, W. Seinen, J.L.M. Hermens,. (1996). *Anal. Chem. , 68*, 4463-4467.

83. E. Urrestarazu Ramos, S. N. Meijer, W. H. J. Vaes, H. J. M. Verhaar, J. L. M. Hermens,. (1998). *Environ. Sci. Technol. , 32*, 3430-3435.

84. Wennan Zhao. (2008). *Solid Phase Microextraction in Aqueous Sample Analysis.* PhD thesis, University of Waterloo, Cheimistry.

85. O.P. Togundea, K. Oakesb, M. Servosb, J. Pawliszyn. (2012). *Anal. Chim. Acta , 742,* 2-9.

86. X. Zhang, K.D. Oakes, M.E. Hoque, D. Luong, C.D. Metcalfe, J. Pawliszyn, M.R. Servos. (2011). *Anal. Chem. , 83*, 3365-3370.

87. H. Yuan, R. Ranatunga, P.W. Carr, J. Pawliszyn,. (1999). *Analyst , 124*, 1443-1448.

88. A.G. Oomen, P. Mayer, J. Tolls. (2000). *Anal. Chem. , 72*, 2802-2808.

89. M.B. Heringa, C. Hogevonder, F. Busser, .J.L.M. Hermens. (2006). *J. Chromatogr. B , 834*, 35-41.

90, F.D. Kopinke, A. Georgi, K. Mackenzie. (2001). *Acta Hydrochim. Hydrobiol. , 28*, 385-399.

91, M.A. Jeannot, F.F. Cantwell. (1997). *Anal. Chem , 69*, 2935-2940.

92. C. Nicholson, J.M. Phillips. (1981). *J. Physiol. , 321*, 225-257.

93. S.W. Lloyd, J.M. Lea, P.V. Zimba, C.C. Grimm. (1998). *Wat. Res. , 32*, 2140-2146.

94. H.H. Jeleń, M. Majcher, R. Zawirska-Wojtasiak, M. Wiewiórowska, E. Wąsowicz. (2003). *J. Agric. Food Chem. , 51*, 7079-7085.

95. Varian 4000 Software Operation Manual. (2004-2009). Version 6. USA.

96. EPA 40 CFR part 136, A. B. (n.d.).

97. R.P. Jones, J.U. Clarke. (2005). *ERDC/TN EEDP-04-36*.