CHAPTER 1

INTRODUCTION

1.1 Background

Usnic acid is a yellowish pigment produced by the fungal partner found in lichens, a mutual symbiosis of fungal and photosynthetic partner which is usually algae. Usnic acid is used mainly in traditional herbal medicine and was known for its antimicrobial properties. However, in the past decades, usnic acid was incorporated into the weight loss supplement in a small dosage related to the effectivity of usnic acid in reducing weight (Guo *et al.,* 2008). Within in vivo testing in rats, usnic acid consumed at low concentration (<500 mg/kg of body weight) could be helpful to reduce body weight because of the capability to do uncoupling oxidative phosphorylation in the mitochondria by decreasing the efficacy of energy use thus increasing thermogenesis (Moreira *et al.,* 2013). Therefore, usnic acid becomes one of the main ingredients for weight loss supplements. The most known brand of the weight loss supplement was LipoKinetix. In 2000, however, few healthy patients upon digesting the weight loss supplement develop acute hepatitis followed by a severe liver injury resulting in liver transplant (Guo *et al*., 2008). It was found that a high concentration of usnic acid found in LipoKinetix had been found to use an excess dosage of usnic acid (>2000 mg/kg of body weight). Excess dosage of usnic acid appears to be a very prominent hepatotoxin that can cause severe liver injury (Moreira *et al.,* 2013).

Detection of usnic acid was performed by Cansaran *et al*. (2007) using a reversed-phase High Pressure Liquid Chromatography (HPLC). This literature for usnic acid detection was chosen to be replicated for the reason that this methodology had the most suitable HPLC conditions to be used in the current laboratory based on the availability of the material and equipment. The method implies the utilization of methanol and phosphate buffer for the elution mobile phase and acetone for the standard diluent with the usage of c18 column and a 20 µl of injection volume along with 0.8 ml/min of flow rate and Photo Diode Array detection. Upon replicating the HPLC conditions, several problems were encountered that affect the chromatogram result for this project. One of the main

problems includes the difference in the column length between the journal and the provided column in the laboratory with the same dimensions between the literature and the availability on the lab in regards to the column diameter and particle size. The column length used by Cansaran *et al.* (2007) was 250 mm while the availability in the lab is 150 mm. The other problem that was encountered is the solvent choices for the usnic acid standard due to the disparity in the solvent strength between the mobile phase and diluent used in Cansaran *et al*. (2007) methodology that disrupted the chromatogram result. The changes of the usnic acid diluent are based on the solubility of the usnic and the solvent strength of the diluent. The changes in mobile phases were based on the improper development of the phosphate buffer during the thesis research due to the minimum information stated within the development of phosphate buffer. Improper formulation of phosphate buffer when mixed with a high concentration of organic solvent induces the increased rate of salt precipitation that disrupt the detection of usnic acid in the HPLC system. The changes in the mobile phase and diluent of the standard would inherently cause the changes in the wavelength spectrum to minimize the diluent interruption in the detectors of HPLC system and the comparison of elution system was done to find the optimum elution system for usnic acid detection along with the changes in injection volume to prevent sample overload in the column that inherently affecting the peak quality and shape in the chromatogram.

The first solution was to convert the optimal flow rate to compensate for the difference in the column length. Several flow rates were tested to find the most efficient flow rate for optimum separation of the compound in a shorter column from a slower flow rate to a faster flow rate in comparison to the original literature flow rate. The utilization of a stronger solvent strength diluent for the usnic acid standard while a weaker solvent strength solvent was used as a mobile phase tends to disrupt the retainability of the targeted compound in the column thus resulting in a bad chromatogram. The diluent was changed into a lower strength elution compound to prevent the disparity of solvent strength and optimize the chromatogram result of HPLC. To compromise with the insolubility of usnic acid in a more polar compound, the concentration of usnic acid standard

was lowered to prevent disruption from the insoluble compound during HPLC analysis. The mobile phase was changed to water: organic solvent (methanol or Acetonitrile) due to the improper development of phosphate buffer used as the mobile phase and the wavelength was changed through a spectrophotometer method to find the best absorbance of usnic acid and prevent the disruption of usnic acid diluent when HPLC analysis was done. The injection volume of the HPLC system determines the volume of the sample injected into the column. The injection volume is one of the most unnoticeable parameters that was rarely changed during the method validation, but the injection volume regulates the optimum volume to be injected into the column to prevent overloading of the sample in the HPLC column for better peak area and height result. Therefore, due to the many problems found in the usnic acid detection methodology based on Cansaran *et al.* (2007) with the usage of HPLC coupled with the difficulty in finding a recent methodology using more advanced analytical equipment, the author decided to optimize the methodology of usnic acid detection based on the errors found in the Cansaran *et al*. (2007).

1.2 Objectives

The objective of this research is to optimize the detection of usnic acid with the usage of reverse phase HPLC system with UV-vis detection with the variation of elution mode and many parameters including the changes of flow rate, mobile phase, usnic acid standard diluent, wavelength, and injection volume.

1.3 Research Significance

This research could open a new opportunity for a simpler method in screening usnic acid in both food and non-food products. The result of the research could be used as a base for the preliminary reference for future method development.

1.4 Outline of Subsequent Chapters

The outline of subsequent chapters is the following: A literature review (Chapter 2), Methodologies (Chapter 3), Results (Chapter 4), Discussions (Chapter 5), and conclusion and Recommendations (Chapter 6), In literature review, fundamental concept were introduced as the following subchapters :

- Usnic Acid
- Implementation of Mitochondrial Phosphorylative Oxidation Uncoupling Properties for Dietary Supplementation
- Beneficial and Adverse Effect of Usnic Acid in Commercialized Product
- Product Containing Usnic Acid
- Usnic Acid Detection
- HPLC basic mechanism
- HPLC factors to Obtain a Good Result

In the methodology, the literature methodology was replicated to test if the method was suitable with the availability of the material and equipment in the laboratory. Several errors were found with the changes of the parameters based on the error founds in the literature methodology. The changes includes: Changes in flow rate, mobile phase and wavelength alteration, Isocratic and Gradient elution, solubility of the usnic acid, injection volume changes. In discussion, the result was further explained with the comparison between the literature review and troubleshooting found in the result. In conclusion and recommendations, all key findings were summarized and suggestions were made to improve the current research. The last part is references provided along with attached appendices as the supplemental raw data of the result.