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Development of an analytical method for the determination of the position specific ^{13}C isotopic composition of organic acids

A partial fulfillment of the requirements for the degree

DOCTOR OF ENGINEERING

By

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Abstract

Stable isotope analysis has been used as a tool to obtain information of origins and histories of organic substances. By detecting the randomly distribution of isotopes in target compounds as an isotope signature, the information about physical, chemical and metabolic processes associated with specific isotope transformation can be revealed. Commonly, compound-specific isotope analysis (CSIA) is the technique that mainly used to analyse the isotope signature of whole molecule of target compound. Because of some limitations in technical usage, the difficulties to access isotope signature of specific atom position have been occurred. Alternatively, position-specific isotopic analysis (PSIA) appears as a better tool to overcome these difficulties and used for investigate isotopic signature of target samples, especially in plants, which can also be applied for other purposes (i.e. food adulteration detection). However, PSIA is undeveloped analytical technique. This study focuses on the development of PSIA for ^{13}C determination of pyruvate, the center organic metabolite, which is important to trigger the plant metabolic system (i.e. citric acid cycle). First, the method for position-specific ^{13}C determination of acetic acid has been developed, as it has the closed relationship to pyruvate by several metabolic processes. After that, this method was adapted for acetic acid derived from pyruvate degradation by H_2O_2. The results have confirmed the successful in method development as pyruvate ^{13}C intramolecular distribution patterns were obtained. This method was later applied to pyruvate from supplementary pills as an application.

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Chapter 1

Introduction

1.1 Organic acids

1.1.1 General

Organic acids are acidic organic compounds, which appear to be the most important intermediate to trigger the metabolism systems in living organisms. Especially in plants, which organic acids play important roles at the cellular level, act as important substrates or intermediates for various biochemical pathways, energy production processes and also the biosynthesis of other organic compounds (i.e. amino acids, fatty acids). Organic acids are biologically obtained through the various metabolic pathways in living organisms and also obtained as products (including by-products) from chemical synthesis processes. The most important organic acids' cycle in plant is the citric acid cycle (or so-called Krebs's cycle), which is the main function for plant respiration system (figure 1-1). Their properties are associated with functional groups (i.e. carboxyl, hydroxyl, etc.…). The most common organic acids are the carboxylic acids, such as formic acid and acetic acid, which their acidity is associated with carboxyl group (-COOH).

1.1.2 Applications of organic acids

Organic acids are commonly used for food applications. Organic acids are also well-known additive for food preservation associated with their antibacterial property [1,2]. They are also used as ingredients in food (i.e. acetic acid in vinegars, ethanol in alcoholic beverages, etc.…). Nowadays, the important organic acids can be purchased in commercial

supermarket in diet supplementary form, depend on people's different purposes. Rather than biologically process such as photosynthesis, organic acids can be either produced by chemical synthesis as an alternative choice of production processes. Especially for massive products, for example: food seasoning or beverages have high demand from consumers around the world. To be competitive, the price of raw materials and production processes are the main factors for manufacturers to make the suitable balance in world market. From this, the analytical methods, which are able to trace raw materials' origin of consumed products, is necessary for human's health and safety at present.

1.2 Isotopes

1.2.1 Background

Isotopes are the various forms of chemical element, which have the same atomic number (same number of protons) but different in atomic weight (different number in neutrons). For example, carbon has three isotopes, which are ^{12}C, ^{13}C and ^{14}C (mass numbers 12, 13 and 14 respectively) with the same atomic number 6. Isotopes can divide into two categories as stable and radioactive (unstable) isotopes. In this study, the stable isotope analysis is the key tool to achieve our objectives, which we focused on stable carbon isotopes. Carbon isotope analysis is one of the methods that used to assess the botanical and geographical origins of target compounds, and thus to detect adulteration in food industry [3-6].

1.2.2 Notation

Isotope ratios (R) are partition of heavy isotopes against light isotopes. Isotopes compositions (δ) can be determined by measuring of

isotope ratios of target sample (R_{sample}), which later standardized by measuring against isotope ratio of standard ($R_{standard}$). The isotope ratio is typically presented in delta notation and expressed in unit "per mil"(‰) as following equation[7-8],

$$\delta = [(R_{sample}/R_{standard})-1] \quad (1)$$

For example, carbon has two stable isotopes[8], which are ^{12}C and ^{13}C with natural abundance percentage at 98.9% and 1.1% respectively. Carbon isotope composition can be determined by the measurement of isotope ratio ^{13}C against ^{12}C (R) of sample against standard (the international standard, Vienna PeeDee Belemnite for carbon) that expressed by the following equation,

$$\delta^{13}C = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard})-1] \quad (2)$$

$\delta^{13}C$ values are commonly expressed in per mil unit (‰). The negative value of $\delta^{13}C$ expresses lighter isotopic composition than standard. For example, plant metabolites or organic compound are ^{13}C depleted compared with standard because plants discriminate against ^{13}C during photosynthesis. The different of isotope content in those organic compounds is related to $^{12}C/^{13}C$ isotope effects.

Chemical reaction, that converts substrate into products, are associated with isotope effect (α), which can be defined by following equation,

$$\alpha = {}^{12}k/{}^{13}k \quad (3)$$

Generally, isotope effect has been categorized into two types [7]. First is kinetic isotope effect, which commonly larger than 1, due to chemical bond interaction (k is rate constant). Second is the effect from thermodynamic, which commonly smaller than 1, due to the free energy of substrate or product that favor or against the heavy isotope (k =

equilibrium constant). The reversible processes are associated with both types of isotope effect.

Common instrument that used for measuring the isotope ratio is Isotope ratio mass spectrometer (IRMS), the special type of mass spectrometer, which used to measure the relative abundance of isotopes in target sample (figure 1-2).

1.3 Isotope analysis techniques

1.3.1 Background

Since the first high-precision isotope ratio mass spectrometry (IRMS) was developed in year 1940[9], the number of research about instrumentation and methods for determination of isotopes of organic elements is rapidly increased. The duel-inlet system that developed in 1940 was the standard for high-precision measurements, requiring the microgram size of target sample and 10-20 minutes for analysis. In a decade of 1970, the use of continuous flow (CF) system that consists of gas chromatograph (GC) with mass spectrometry (MS) was begin [10,11]. Then later in 1980, high precision system consists of element analyzer interface with IRMS for ^{13}C determination was developed [12] and use as the worldwide method for study field such as ecology, geochemistry, and biomedicine. For isotope analysis, the samples must be introduced to instrument as a specific gas (i.e. CO_2 for carbon isotope analysis), which also requiring conversion process while maintaining isotopic signature of the original sample. Due to the popular subjects of research, the natural samples such as atmospheric CO_2, plants and animals were used. Since the conversion process of these chemically complex samples required the cumbersome processes (i.e. preparative separation from whole sample, etc.…), uncontrolled isotopic fractionation and contamination is become

the significant problem occurred by these procedures. Some studies were done with the more additional complex step of chemical isolation to confirm the uncontaminated form of samples before isotope analysis [13-15]. In the decade of 1990, the development concept of GC-combustion-IRMS (GC-C-IRMS), using online separation system for compound specific isotope analysis (CSIA) has been introduced to use in isotope analysis. The developed system help the user reduce the contamination and help characterized the produced isotope fractionation [16,17]. This concept was lately extended to liquid chromatography (LC) for carbon isotope analysis and developed to the LC-IRMS system.

1.3.2 Compound Specific Isotope Analysis (CSIA)

Compound specific isotope analysis (CSIA) is one of the analytical methods for the measurement of isotope composition in target compound. Since isotopic composition in the compounds is the function of their starting materials and production processes as well as the degradation after they were made, CSIA has several applications in environmental studies. For example, using CSIA technique, isotope composition of target samples was obtained and can be used as tracers to its origin (i.e. original area, type of plants, etc.…)[3-6,18-21]. Nowadays, CSIA technique is a very convenient technique and adapted worldwide as a tool for helping in many kinds of works and studies. However, this technique still has its limitation to provide enough information for some works, especially the determination of isotope composition of site-specific carbon atoms in same compounds, which appear to be non-homogeneous pattern. The advanced work required the additional methods to access the information of each atom in same sample or compound.

1.3.3 Position Specific Isotope Analysis (PSIA)

Position specific isotope analysis (PSIA) is a developed technique to overcome the limitation of CSIA technique with more complicate steps. Generally, the study issue of position specific measurements is limited to only the important problems because of the burdensome steps of chemical degradation and separation steps are required before isotope analysis. The very first work of PSIA is Abelson and Hoering in 1961 [13], which isolated the carboxyl position of amino acids, using ninhydrin reaction before its subsequent analysis. The results of this work showed the ^{13}C enriched of carboxyl position relative to the rest of molecule, which is the indirect evidence about the glycolysis proceeds without isotope discrimination. Because of laborious steps of working, the later works had established in a decade later. In 1977, Deniro and Epstein [14] had studied about the precise enzymatic step of pyruvate by determined carbon isotope fractionation at acetate position, which result in the low enrichment of ^{13}C in lipids. With these results, the work about PSIA were continued to study and verified the prediction of carbon isotope pattern in organic acids, fatty acids, etc.[22,23]. Later, the alternative choices for PSIA studies to avoid the difficulties of laborious steps have been developed such as the adaption of nuclear magnetic resonance (NMR) or pyrolysis GC systems [28, 35-37].

(PSIA) appears as a valuable yet-underemployed tool to investigate isotopic fractionation in environmental system such as plants. In addition, position specific isotope analysis (PSIA), i.e. intramolecular carbon isotope analysis, have potential to access the information since the isotopic composition of specific atom can be determined. Heterogeneous isotopic distributions have been shown for diverse organic compounds (amino acids, acetic acid, fatty acids, sugars, ethanol, hydrocarbons...) [25-34]. Although technical barriers make PSIA difficult, these barriers can be overcome by using separation techniques such as quantitative NMR, or

by degrading the molecule into fragments which isotopic composition are then subsequently determined after separation. The interest of the latter relies on the fact that it can be made online with coupling to conventional GC-C-IRMS [37], making it a particularly sensitive method. Yet, the accuracy of offline or online degradation methods is not straightforward and is often difficult to assess. This study will be an important step to learn about further suitable methods for PSIA in important organic acids, which play an important role in Krebs cycle as well.

1.4 Potential for ^{13}C method development of organic acids

Many previous works showed the important of position specific analysis of carbon isotope. In 1991, Rossmann et al. [38] found the non-homogeneous ^{13}C pattern in natural glucose, which can be implied that the data from CSIA is not enough to explain about the mechanism and carbon isotope composition inheritance in specific carbon position of organic compounds. To access the carbon isotope composition of each atom, the suitable analytical method is needed. From the metabolic map in plants (figure 1-1), the center metabolite in plant metabolism is pyruvate. Melzer and Schmidt [23.24] studied and established offline procedure for ^{13}C PSIA of pyruvate. However, the analytical method of pyruvate still required the laborious steps and complicated instruments for chemical degradation and purification, which are time-consuming process and have a risk of sample contaminated. The alternative choices such as some online analytical methods have still not yet established. Considering the disadvantages of the exist method, the development of analytical method associate with the online system that can help to avoid these obstructs can be a good attempting for making the new alternative way of PSIA of pyruvate. Thus, to develop and establish the online analytical method, is necessary for further study. Since the pyruvate is

cornerstone metabolite of the plant system, to obtain the suitable analytical method of pyruvate is the best attempting to obtain the carbon isotope information. Later, the successful in pyruvate method will benefits for adapt and extend to develop the analytical method for other organic acids in the future.

1.5 Study objective

The main objective of this study is to develop the analytical method, that is helpful for CSIA and PSIA, for clarify the carbon isotope distribution and fractionation, which are related to organic acids by using the information of carbon isotope composition. In order to that, the development of the analytical method of carbon isotope composition in organic acids is needed. In this study, we focus on development of analytical method of pyruvate, which is the most important metabolite to initiate the plant's citric acid cycle for respiration system (figure 1-1). This study is consists of the improvement of the exist analytical method that is discussed further in chapter 2 and adaption of this developed method to make the analytical method of bigger organic acids molecule in chapter 3. First of all, the analytical method for $\delta^{13}C$ of organic acids have been developed, started from acetic acid, which is one of the most important intermediate and the simplest organic acids with two carbon atoms. With the success in the developed analytical method for acetic acid, then we apply the method to pyruvate. The achievement of pyruvate's developed method will be the important part of further studies in plant's metabolic pathways and also method development in future.

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Tables and Figures

Figures

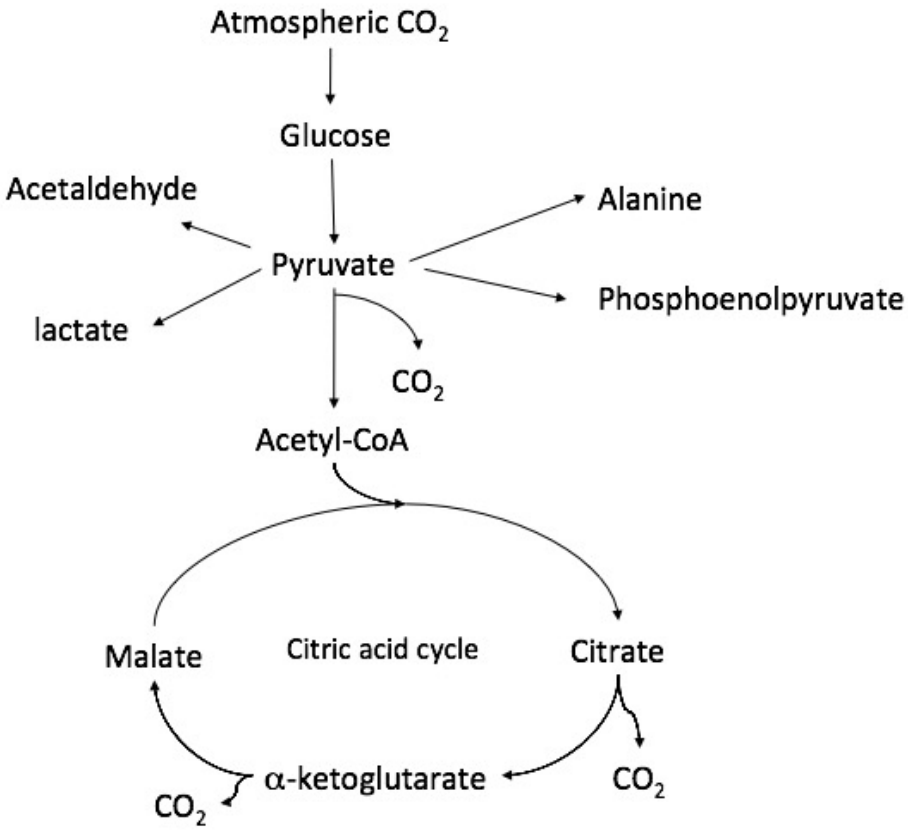

Figure 1-1. From atmospheric CO_2 to plant respiration system

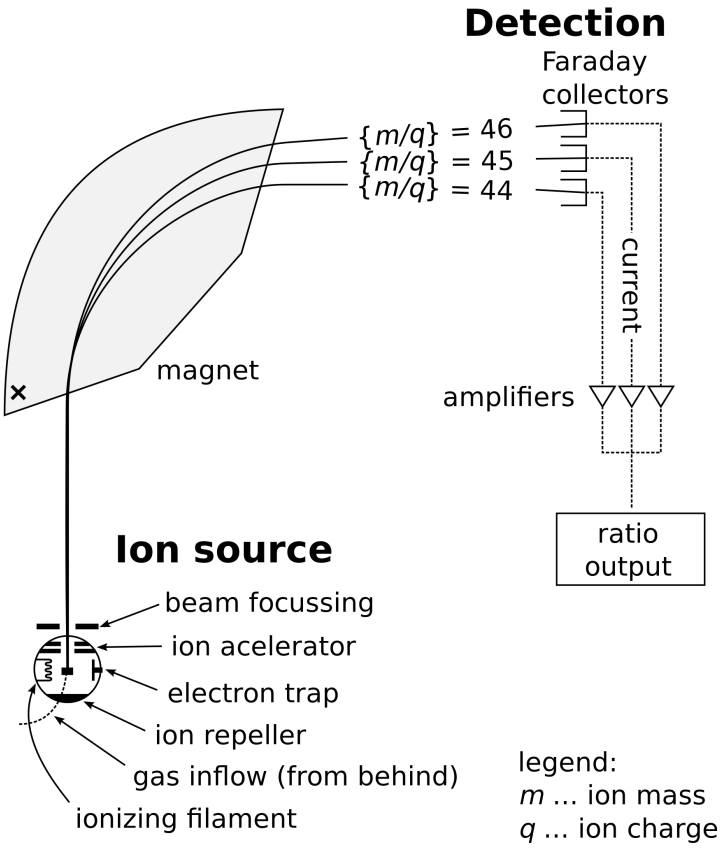

Figure 1-2. Configuration scheme of isotope ratio mass spectrometer, measuring CO_2 (adapted from United States Geological Survey report [39])

Chapter 2

Analytical method for simultaneous determination of bulk and intramolecular ^{13}C isotope compositions of acetic acid

2.1 Introduction

2.1.1 Acetic acid

Acetic acid (CH_3COOH) is one of the simplest carboxylic acids. It has a systematically name as ethanoic acid. Acetic acid can be produced by biological production such as oxidative fermentation of ethanol or chemical synthesis. Among organic molecules, acetic acid represents an important intermediate in metabolism. Analyzing and interpreting its isotope distribution can lead to indications of pathways and fluxes used by organisms in synthesizing it. Additionally, its presence in food materials such as vinegar makes it an interesting proxy for authentication purposes. Attempts have been undertaken to ascertain the intramolecular carbon isotope distribution in acetic acid and its salt using both offline and online methods.

2.1.2 PSIA of acetic acid and previous studies

A previously used method included chemical degradation by pyrolysis, followed by analysis of pyrolytic products using isotope ratio mass spectrometry (IRMS).[10,12,13] To overcome the shortcomings of the offline methods such as time-consuming analysis and a large volume of samples required, online analytical techniques have been developed to analyse the carbon isotope composition of pyrolytic products of acetic acid.[14,15] Recently, Hattori et al.[16] reported a modified online pyrolysis method used for the measurement of intramolecular carbon isotope

composition of acetic acid in vinegars using a solid phase micro extraction (SPME) device along with gas chromatography – pyrolysis-gas chromatography –combustion – isotope ratio mass spectrometry (GC-Py-GC-C-IRMS). Acetic acid was pyrolyzed and degraded into pyrolytic products such as CO, CH_4, CO_2 C_2H_6, and C_3H_8 before analysis using the GC-C-IRMS system. The carbon isotope composition of pyrolytic CO_2 is used for the determination of the carbon isotope composition of the carboxyl component (-COOH) of the original acetic acid. The carbon isotope composition of the remaining methyl part (-CH_3) can be calculated using a mass-balance equation integrating the carbon isotope composition of the carboxyl part and of the original acetic acid molecule. Nevertheless, the technique requires two separate analyses using two configurations, GC-C-IRMS and GC-Py-GC-C-IRMS systems (figure 2-1, 2-2) to characterize a molecule, which are a time-consuming process and a potential additional source of error.

2.1.3 Aim of study

This study aims to develop a novel analytical method that enables the simultaneous determination molecular and intramolecular carbon isotope composition of a compound using only configuration of a GC-py-GC-C-IRMS system without further need to alter the instrument configuration. We then applied this method for determination of the carbon isotope distribution of acetic acid in commercial vinegars.

2.2 MATERIALS AND METHODS

2.2.1 Notation

2.2.1.1 Carbon isotope ratio

The carbon isotope composition in per mil (‰) is expressed as $\delta^{13}C$ value, the carbon isotope ratio ($^{13}C/^{12}C$) of the sample against an international standard (VPDB).

$$\delta^{13}C = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard})-1] \quad (1)$$

2.2.1.2 Bulk and intramolecular $\delta^{13}C$ of acetic acid

Bulk and intramolecular $\delta^{13}C$ of acetic acid can be defined in a mass balance equation as follows,

$$\delta^{13}C_{AcOH} = (\delta^{13}C_{CH3} + \delta^{13}C_{COOH})/2 \quad (2)$$

Where $\delta^{13}C_{AcOH}$ is the bulk carbon isotope composition of acetic acid; ($\delta^{13}C_{CH3}$ and $\delta^{13}C_{COOH}$ denote carbon isotope composition of methyl and carboxyl carbon atom of acetic acid, respectively.

2.2.2 Chemicals

2.2.2.1 Acetic acid standards

Five acetic acid standards and three sodium acetate standards[17] designated as AAA (Wako Pure Chemical Industries Ltd., Osaka, Japan), AAB (Aldrich, Milwaukee, WI, USA), AAC (Aldrich, Milwaukee, WI, USA), AAE (Sigma-Aldrich Corp., St. Louis, MO, USA), AAF (Nacalai Tesque Inc., Kyoto, Japan), SAA (Wako Pure Chemical Industries Ltd., Osaka, Japan), SAB (Kanto Chemical Co. Inc., Tokyo, Japan), and SAC (Aldrich, Milwaukee, WI, USA) were used for this study.

2.2.2.2 Vinegar samples

Nine vinegar samples commercially available in Japan were used for this study. Their acetic acid contents were 5–7%, according to their respective package labels.

2.2.3 Instruments

2.2.3.1 GC-Py-GC-C-IRMS system

A GC-Py-GC-C-IRMS system (figure 2-2) was used for intramolecular $\delta^{13}C$ analysis. It consists of a first gas chromatograph, (Trace™ GC Ultra; Thermo Fisher Scientific Inc., Bremen, Germany) equipped with a capillary column (Nukol™, 30 m × 0.32 mm i.d., 1 μm film thickness; Supelco, PA, USA), connected to a second gas chromatograph (HP 6890 series; Hewlett-Packard Co., PA, USA) equipped with a second capillary column (HP-Plot Q 30 m × 0.32 mm i.d., 20 μm film thickness; Agilent Technologies, CA, USA). The two gas chromatographs were connected through a pyrolysis furnace part (ceramic tube, 25 cm× 0.5 mm i.d.), operated at 1000 °C for pyrolysing acetic acid. The pyrolytic products were separated by second capillary column and introduced into a combustion furnace (ceramic tube, 25 cm× 0.5 mm i.d., packed with CuO, NiO and Pt wires), operated at 960°C. The second chromatograph was connected via Thermo GC Isolink™ and Conflo-IV™ interfaces (Both are from Thermo Fisher Scientific Inc.) to a mass spectrometer (Finnigan Delta V™, Thermo Fisher Scientific, Inc.). A transfer line between chromatographs was made using deactivated fused silica capillary (0.32 mm i.d.; GL Sciences, Japan).

2.2.3.2 Solid phase micro extraction (SPME)

Solid phase micro extraction is a device equipped with fiber coat with extracting phase such as liquid (polymer), solid (sorbent) or a combination of both phases (figure 2-3). The device acts as a containing of target sample after extraction in the same manner as common syringe device. After extraction, SPME fiber is inserted directly to the chromatograph for subsequent analysis.

2.2.4 Sample preparation and extraction

2.2.4.1 Sample preparation

For this study, all acetic acid samples (standards and vinegar samples) were prepared at 85 mM by dilution with distilled water. First, 1 mL of samples was put in in 20 mL gas-tight vial, topped with rubber cap for subsequent analysis. The sample pH was adjusted to 1.0–2.0 pH by adding 0.2 mL of 0.1 mol/L HCl.

2.2.4.2 Acetic acid extraction by SPME

Acetic acid from samples was extracted from the headspace (HS) using a solid-phase micro extraction (SPME) device, equipped with 85 μm thickness SPME fiber coated with carboxen/polydimethylsiloxane (Carboxen/PDMS stableflex™; Supelco, PA, USA).[16] Extraction was conducted in a thermostatic chamber controlled to 25 °C, non-stirred samples . The extraction time was 60 min.

After HS-SPME extraction, the fiber was inserted to the injection port of the first gas chromatograph at 250 °C. Helium was used as a carrier gas in all experiments. Chromatographic conditions were the following:

2.0 mL/min flow rate of carrier gas and 10:1 split ratio. The first oven temperature program was the following: 100 °C (5 min), then increased to 190 °C (10 min) at the rate of 15 °C/min, and finally increased to 200 °C (2 min) at the rate of 15 °C/min. The second gas chromatograph was kept constantly at 40 °C.

2.3 RESULTS and DISCUSSION

2.3.1 Calibration curve and calculation of $\delta^{13}C$ of acetic acid

Pyrolytic products detected using the method presented in the Materials and Methods section are the following, in order of elution: CO, CH_4, CO_2, C_2H_6, and C_3H_8. Carbon dioxide has already been shown to arise solely from the carboxyl atom position of acetic acid.[14] Assuming filiation between the original acetic acid molecule and the pyrolytic products, then the following formula can be inferred.

$$CH_3\text{-}COOH \rightarrow CH_4 + CO_2 \quad (3)$$

The intramolecular isotope composition can be calculated from the $d^{13}C$ values of CH_4 and CO_2. To estimate the potential extent of C-exchange and the isotope fractionation factors associated with the process, we determined $\delta^{13}C_{CO2}$ and $\delta^{13}C_{CH4}$ for eight working isotopic standards samples for which the intramolecular isotopic composition has already been determined in a previous study using off-line pyrolysis.[17] They are expected to follow the equation shown below.

$$\delta^{13}C_{CH3} = a_{methyl}.\delta^{13}C_{CH4} + b_{methyl} \quad (4)$$

$$\delta^{13}C_{COOH} = a_{carboxyl}.\delta^{13}C_{CO2} + b_{carboxyl} \quad (5)$$

Therein, $\delta^{13}C_{COOH}$ and $\delta^{13}C_{CH3}$ respectively denote isotope compositions for the carboxyl and methyl components of standard acetic acid; $\delta^{13}C_{CO2}$

and $\delta^{13}C_{CH4}$ respectively (equation 2) represent the isotope composition of the pyrolytic products of acetic acid pyrolysis CO_2 and CH_4; *a* is a parameter representing the fidelity of the filiation between starting acetic acid and pyrolytic products; *b* is a parameter representing isotope fractionation and also the $\delta^{13}C$ value-shift of reference gas (CO_2) associated with the overall analytical process. Table 2-1 presents the relation between $\delta^{13}C$ values of isotopic standards and $\delta^{13}C$ values of pyrolytic products. The $\delta^{13}C$ value of the reference gas (CO_2) was set to 0‰ arbitrarily. Parameters obtained from these curves are shown in table 2-1.

The slope of correlation lines (carboxyl and methyl components) shows the $\delta^{13}C$ values of pyrolytic products (figure 2-4). The certified $\delta^{13}C$ value relation fits the 1:1 trend perfectly, which implies high fidelity of the filiation between pyrolytic products and starting material. In other words, CO_2 arises only from the carboxyl part; CH_4 arises only from the methyl part of the parent acetic acid. The *y*-intercept value is used to calculate the $\delta^{13}C$ values of methyl and carboxyl positions of acetic acid. The standard errors (n=8) on the y-intercept are, respectively, 0.31‰ and 0.24‰ for the methyl and carboxyl parts. Given that the standard deviation of the $\delta^{13}C$ values of CH_4 and CO_2 products are 0.38‰ and 0.40‰, the overall standard deviation for the calculation of $\delta^{13}C$ values of CH_3 and COOH parts of acetic acid is below 0.6‰, which is acceptable when compared with previous reports aiming at determining intramolecular isotope composition.

Overall, the most important feature of the present approach is that switching between the configurations of the two systems is unnecessary, which is a benefit both in terms of the experimental time and in terms of technical maintenance.

2.3.2 Intramolecular and molecular $\delta^{13}C$ values of acetic acid in vinegar

Molecular and intramolecular $\delta^{13}C$ values of acetic acid in nine vinegar samples were ascertained using the developed method. The maximum standard deviations (n=3) are, respectively, 0.5‰ and 0.3‰ for $\delta^{13}C_{COOH}$ and $\delta^{13}C_{CH3}$. The isotopic composition of acetic acid molecule ($\delta^{13}C_{AcOH}$) was also calculated using the mass balance equation of acetic acid as $\delta^{13}C_{AcOH} = (\delta^{13}C_{carboxyl} + \delta^{13}C_{methyl})/2$. Vinegars show intramolecular $\delta^{13}C$ distribution in similar patterns with $\Delta\delta$ ($\delta^{13}C_{COOH} - \delta^{13}C_{CH3)}$ values positive for all samples (average $\Delta\delta = 4.3 \pm 2.0$ ‰) except vinegar B that show the negative $\Delta\delta$ value around -2.8 ‰ (table 2-2). These values must be placed in the context of biosynthetic isotope fractionation associated with acetic acid production and isotope composition of the starting material. The negative of $\Delta\delta$ value in sample B might depends on the different of raw materials or production process. However, this trend cannot be making the concrete conclusion yet, due to lacking of information at the moment. Vinegars are produced by fermentation of alcoholic beverages, during which ethanol is oxidized to acetic acid. The intramolecular isotope composition of ethanol is a key determinant of the isotope composition of acetic acid. The intramolecular isotope distribution in ethanol from different origins has been explored in past decades, either by chemical means or by isotopic quantitative ^{13}C NMR, and is now well documented.[8,18–22] Briefly, the $\delta^{13}C$ values of methyl and methylene carbon positions in ethanol are inherited, respectively, from that of C-1/C-6 and C-2/C-5 carbon atom positions of hexoses. Because the intramolecular distribution of ^{13}C in hexoses is

governed by the CO_2 assimilation mechanisms of plants from which they are extracted (C_3, C_4, CAM), differences arise in the isotopic distributions of ethanol. The differences in $\delta^{13}C$ values between CH_2OH and CH_3 positions are 3–4‰ for C_3 plants, 0–2‰ for C_4 plants and ethanol from CAM plants exhibit the highest gap (up to 10‰).[16] This gap is, at least partly, responsible of the ^{13}C-enrichment observed in the COOH position of acetic acid analysed in this study. Isotope fractionation associated with the oxidation of ethanol to acetic acid must also play a role in the isotopic distribution of the product, although less is known about it. Rinaldi et al.[13] reported ^{13}C-depletion on the methyl site of acetic acid formed from ethanol oxidation by *Acetobacter suboxydans*, the $\delta^{13}C$ value of the carboxyl position remaining unchanged. Scharschmidt et al.[23] demonstrated that the oxidation of benzyl alcohol or benzaldehyde by alcohol dehydrogenase and aldehyde dehydrogenase is associated with an ^{13}C isotope effect of 1.012–1.025 on the oxidized position of products. Transposing this to acetic acid production from ethanol engenders depletion of the carboxyl position through the oxidation of ethanol to acetic acid. Although these studies might seem contradictory at first sight, it must be borne in mind that the former used cultured organisms whereas the latter used pure enzymes. Therefore, in the former case, additional metabolic factors such as commitment of ethanol and acetic acid to other metabolic reactions, and uptake of ethanol through the membrane, might play a role in the isotope distribution of acetic acid. In addition, vinegar samples analysed in this work are from commercial local stores, which imply that their origin cannot be ascertained. Therefore, further studies on isotope fractionation under controlled conditions would be valuable. Systematic studies must be conducted to constrain isotope fractionation associated with transformations (e.g. fermentation) or enzymatic reactions (e.g. aldehyde dehydrogenase). The method presented here is

expected to be of great help for this purpose. Results from such experiments will be addressed in a subsequent study.

2.4 CONCLUSION

The HS-SPME-GC-Py-GC-C-IRMS method developed for this study is useful to obtain $\delta^{13}C$ of acetic acid at both intramolecular and molecular levels in a single analysis. Results show the analysis of intramolecular $\delta^{13}C$ distribution of commercial vinegar samples with ±0.6‰ repeatability. From these results, we can avoid switching between two-configuration system and twice of sample preparation. In parallel, to avoid twice of sample preparation can reduce the amount of sample to use and also benefits about preparation time for the real sample that need the complicate purification steps. This study also shows the importance of using intramolecular isotope standards to calibrate novel approaches.[17,24] The convenience of this developed method will be beneficial in order to spread the technique and thus to expand potential applications. The approach presented herein enables future development for the determination of intramolecular isotope composition of important metabolites (pyruvate, malate, etc.). These future developments will be described in later reports.

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distribution in organic molecules by isotopic ^{13}C nuclear magnetic resonance spectrometry. *Anal. Chim. Acta* **846**, 1-7.

Tables & Figures

Tables

Table 2-1. Parameters obtained from the linear regression of the curve representing position-specific isotope composition of previously measured standards and pyrolytic fragments measured in this study for methyl and carboxyl C-atom positions of acetic acid.[17]

y=f(x)	Slope (a)	Intercept (b)	R^2
$\delta^{13}C_{CH3} = f(\delta^{13}C_{CH4})$	0.99±0.02	30.40±0.31	0.994
$\delta^{13}C_{COOH} = f(\delta^{13}C_{CO2})$	0.99±0.03	29.04±0.24	0.999

Table 2-2. $\delta^{13}C$ distribution of nine commercial vinegars from different raw material sources. "Raw materials" are those written on the packaging label.

Vinegar	Raw materials	$\delta^{13}C_{COOH}$ (‰)	$\delta^{13}C_{CH3}$ (‰)	$\delta^{13}C_{AcOH}$ (‰)	$\Delta\delta^{a}$
A	organic apple	-20.6 ± 0.5[b]	-27.7 ± 0.3	-24.2 ± 0.4	7.1
B	maize, barley	-13.4 ± 0.5	-19.6 ± 0.2	-12.0 ± 0.4	-2.8
C	malt vinegar from maize, barley	-18.4 ± 0.3	-19.5 ± 0.1	-18.9 ± 0.2	1.2
D	wine, raspberry juice	-26.5 ± 0.3	-29.6 ± 0.1	-28.0 ± 0.3	3.1
E	wine, cherry	-20.9 ± 0.4	-28.0 ± 0.3	-24.4 ± 0.4	7.1
F	wine, herbs	-25.0 ± 0.5	-29.3 ± 0.3	-27.2 ± 0.4	4.2
G	roasted malt from barley	-27.0 ± 0.3	-29.9 ± 0.2	-28.4 ± 0.2	2.9
H	red wine	-24.5 ± 0.1	-28.8 ± 0.1	-26.7 ± 0.1	4.4
I	white wine	-25.4 ± 0.2	-29.4 ± 0.2	-27.4 ± 0.2	4.0

[a]: $\Delta\delta$ (‰)= $\delta^{13}C_{COOH}$ - $\delta^{13}C_{CH3}$

[b]: standard deviation from the mean (n=3)

Figures

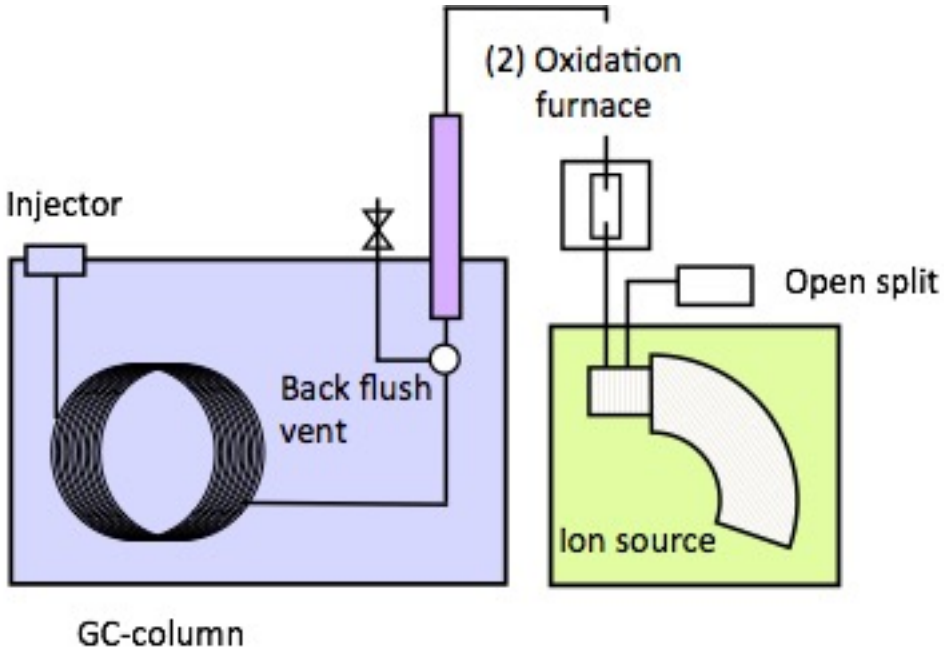

Figure 2-1. Schematic diagram of GC-C-IRMS system

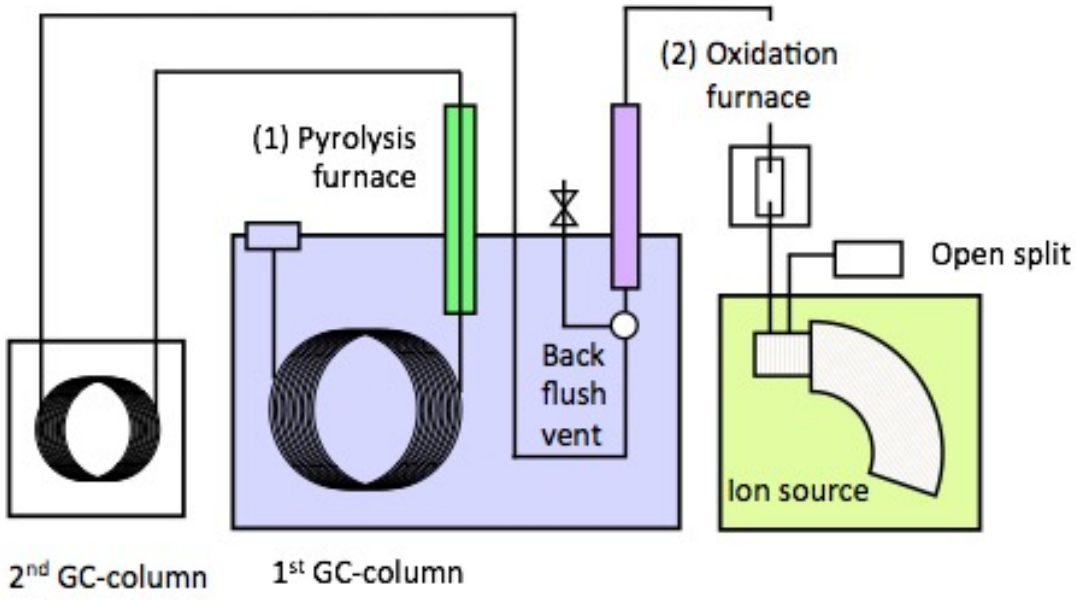

Figure 2-2. Schematic diagram of GC-Py-GC-C-IRMS

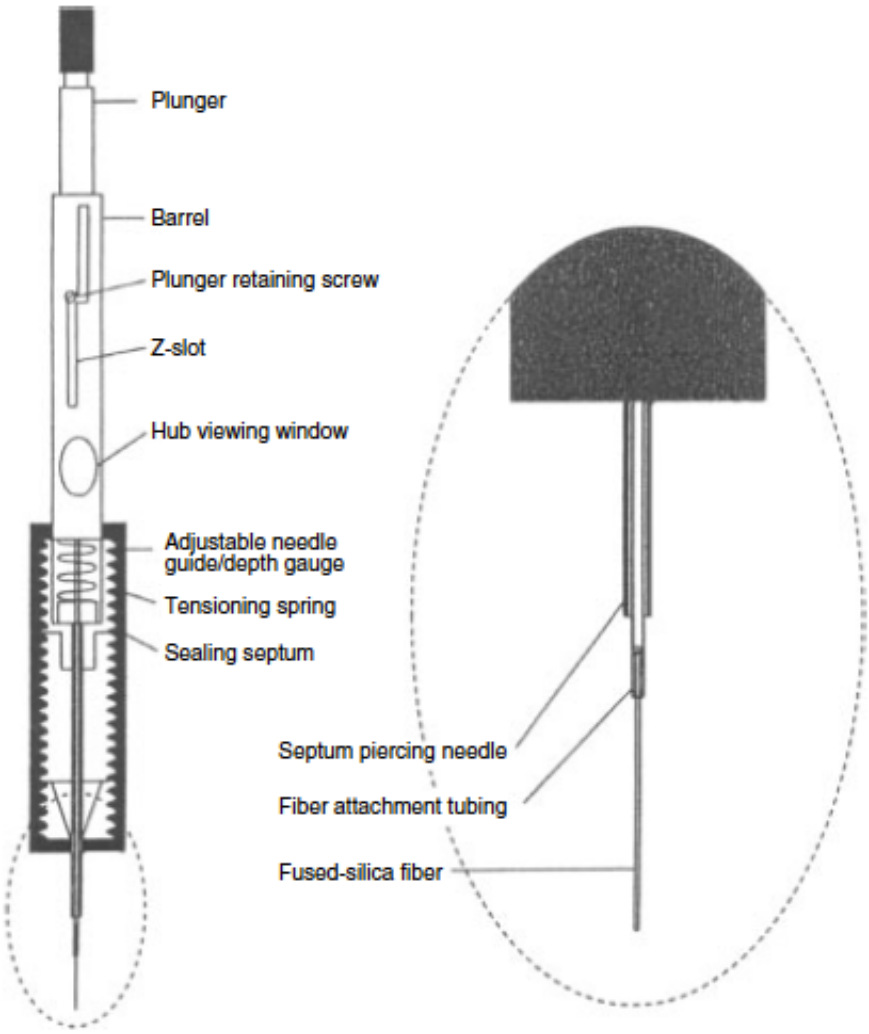

Figure 2-3. Schematic diagram of SPME device (Adapted from Gyorgy Vas and Karoly Vekey *J.Mass Spectrom.* 2004;39:233-254)

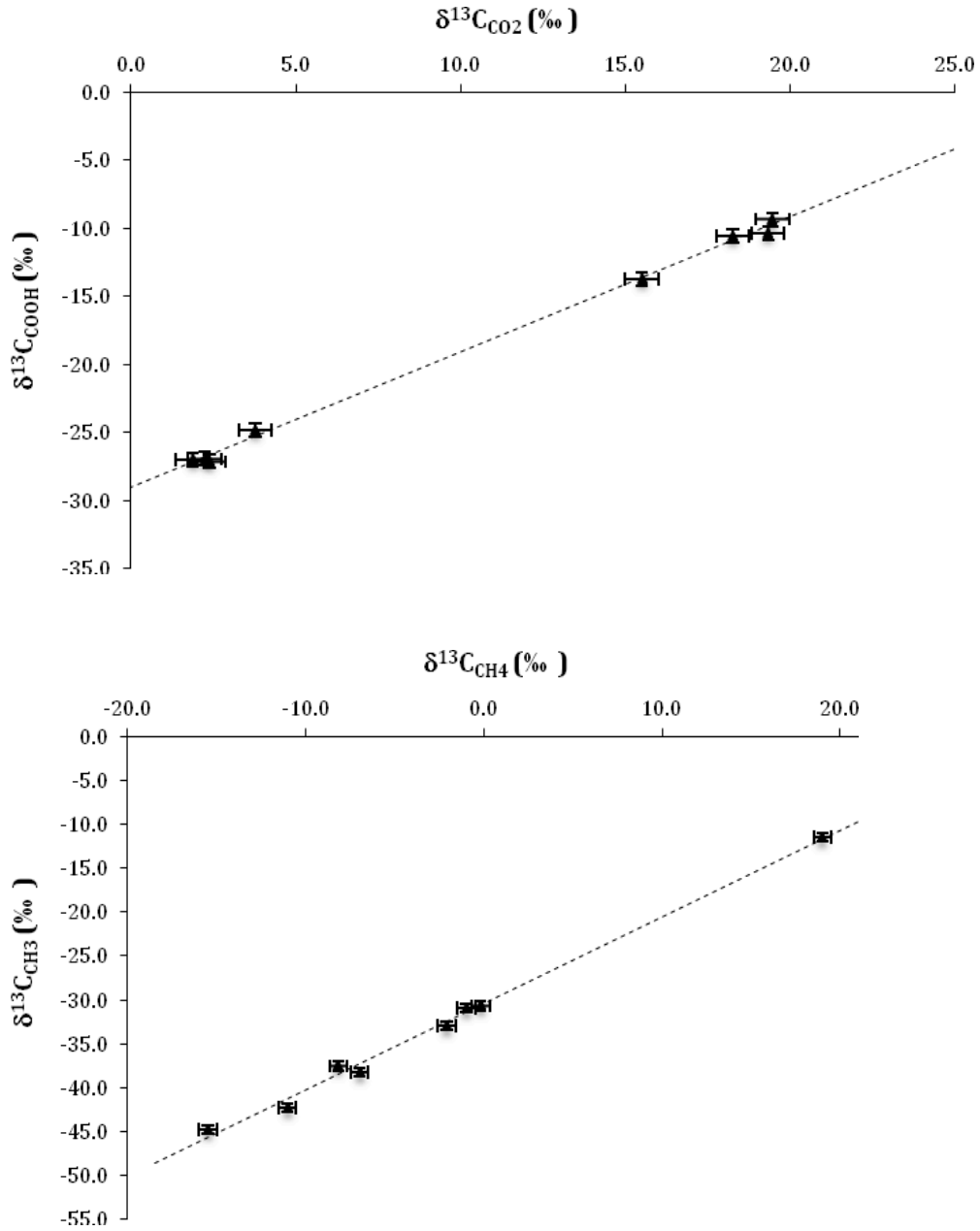

Figure 2-4. Correlation curves for certified intramolecular $\delta^{13}C$ values ($\delta^{13}C_{COOH}$ and $\delta^{13}C_{CH3}$) and those measured using the online method examined in this study ($\delta^{13}C_{CO2}$ and $\delta^{13}C_{CH4}$). The dotted line shows best linear fitting (see text for equations and R^2).

Chapter 3

Determination of intramolecular ^{13}C isotope distribution of pyruvate

3.1 Introduction

Isotope analysis technique has been applied to help identifying the origin, metabolic pathways, and biosphere/atmosphere interaction of organic materials[1-4]. Position-Specific Isotope Analysis (PSIA) has provided information of heterogeneous isotope distribution of organic compounds [5-9]. This information is crucial for the investigation of synthetic processes and metabolic pathways of the target compound. These isotope analysis techniques are also applied in food industry for the purpose of quality control.

3.1.1 Role of pyruvate

Pyruvate (CH_3COCOO-) is a key metabolite for carbohydrate metabolism, which is needed in order to trigger the plant's citric acid cycle, fat, and protein metabolism. It also can be used as a dietary supplement to increase metabolic rate [10]. Since pyruvate influences the isotopic content of respired CO_2 and its related metabolites, its isotope signature would be beneficial in studying authenticity and metabolic pathways in plants. In this study, the main objective is to improve the analytical method for intramolecular ^{13}C distribution of pyruvate, which can be degraded into acetic acid and CO_2 by using H_2O_2 [11,12]. The results confirm the success of this technique as we obtained pyruvate ^{13}C intramolecular distribution pattern.

3.1.2 Pyruvate Supplementary

Additionally, we applied this method to determine intramolecular ^{13}C distribution in pyruvate sample from pyruvate supplement pills. Dietary supplement is one of the convenient choices that provide essential nutrients. Owing to its high demand, the number of manufacturers has been rapidly increased in the past few years. One way to remain competitive in the market is to decrease the manufacturing cost as much as possible. Commonly, people prefer the authentic products from natural, however the same commercial synthetic substance, which can be obtained by the derivatization of petroleum or coal, offers a faster and cheaper alternative than the natural extracted from biogenic sources [13-15]. According to the Food and Drug Administration (FDA) in some countries, synthetic substances are illegal and prohibited from manufacturing [16]. To minimize the risk of illegal action, the method to help differentiate between synthetic and natural substances is needed [17-19]. In this study, we examined the intramolecular ^{13}C distribution of pyruvate in dietary supplement, which indicates the potential origin. This work was compared with previous study[11] regarding the agreement of ^{13}C values and the method efficiency.

3.2 MATERIALS AND METHODS

3.2.1 Notations

The carbon isotope composition in per mil (‰) concentrations is expressed as the $\delta^{13}C$ value, the carbon isotope ratio ($^{13}C/^{12}C$) of the sample against an international standard (VPDB).

$$\delta^{13}C = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard}-1] \quad (1)$$

For this study, pyruvate samples were measured after decarboxylation, which yields acetic acid and carbon dioxide. Bulk and intramolecular

$\delta^{13}C$ values of pyruvate are definable in a mass balance equation as

$$\delta^{13}C_{Pyruvate} = [2(\delta^{13}C_{AcOH}) + \delta^{13}C_{CO2}]/3. \quad (2)$$

Therein, $\delta^{13}C_{AcOH}$ value is the bulk carbon isotope composition of acetic acid and $\delta^{13}C_{CO2}$ value is the carbon isotope composition of CO_2 from pyruvate decarboxylation in this study. Actually, bulk and intramolecular $\delta^{13}C$ values of acetic acid are definable in a mass balance equation as

$$\delta^{13}C_{AcOH} = (\delta^{13}C_{CH3} + \delta^{13}C_{COOH})/2, \quad (3)$$

where $\delta^{13}C_{CH3}$ value and $\delta^{13}C_{COOH}$ value respectively represent the carbon isotope composition of methyl and carboxyl carbon atom of acetic acid. Each carbon position of pyruvate was designated as C-1 (carboxyl part), C-2 (carbonyl part), and C-3 (methyl part).

3.2.2 Chemicals

3.2.2.1 Sodium pyruvate samples

Four sodium pyruvates designated as A (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan), B (MP Biomedicals, LLC, CA, USA), C (Sigma-Aldrich Corp., St. Louis, MO, USA), and D (Wako Pure Chemical Industries Ltd., Osaka, Japan) were used for this study.

3.2.2.2 Pyruvate supplement samples

Four pyruvate supplement samples, designated as DP1 (Earth Natural Supplements, Florida, USA), DP2 (Best Naturals, NJ, USA), DP3 (Source Naturals, Inc., CA, USA) in capsule pills, and DP4 (Now Foods, IL, USA) in tablet pills were purchased.

3.2.2.3 Other chemicals and accessories

Hydrogen peroxide (30%) and hydrochloric acid (0.1 mM) (Wako Pure Chemical Industries Ltd.) were used respectively for pyruvate decarboxylation and pH adjustment. A tin capsule (0.15 ml: ∅ 5/19 mm; LÜDI Swiss AG, Switzerland) was used to contain sodium pyruvate samples for laser spectroscopy analysis.

3.2.3 Degradation of pyruvate

H_2O_2-catalyzed decarboxylation of pyruvate is described according to the following scheme:

$$CH_3COCOOH + H_2O_2 \rightarrow CH_3COOH + CO_2 + H_2O. \qquad (4)$$

In this study, sodium pyruvate samples (A, B, C, and D) were degraded using 30% hydrogen peroxide, then yielding acetic acid, CO_2 and H_2O as products, as described below. The yields of acetic acid at 10, 30, 60, and 120 min of degradation time were determined using ion chromatography (IC-20 Dionex™; Thermo Fisher Scientific Inc., Bremen, Germany).

For the degradation of sodium pyruvate, a pyruvate aqueous solution was prepared at 85 mM diluting with distilled water. For pyruvate supplement samples, pyruvate was separated from other ingredients before dilution with distilled water. One pill of 750 mg (DP2, DP3) and 1000 mg (DP1, DP4) was used for pyruvate extraction. According to packages' label, pyruvate supplementary samples have main ingredients consists of pyruvate salt, gelatin (contained capsule), stearate, and cellulose. The gelatin container was taken off and discarded (DP1, DP2, DP3); the powder sample was kept. The DP4 tablet was crushed to powder in ceramic mortar. Remaining powder of samples was diluted in 100 mL milliQ water. Because of the lack of water solubility, stearate and

cellulose were separated using microfiltration three times using a 20 μm filter. Then the pyruvate aqueous solution was obtained from supplement samples (figure 3-1).

In a 20 mL gas-tight vial, 1 mL of each pyruvate aqueous solution sample was put in and topped with rubber cap for analysis. To samples for pyruvate degradation, 0.2 mL of H_2O_2 was added. After complete degradation, the samples' pH was adjusted to 1.0–2.0 pH by adding 0.2 mL of 0.1 mol/L HCl. The CO_2 derived from the degradation was collected and purified by repeated cryogenic method and trapped in the Pyrex® sealed tube for $\delta^{13}C$ analysis.

3.2.4 Instruments and $\delta^{13}C$ analysis

3.2.4.1 HS-SPME-GC-Py-GC-C-IRMS

Intramolecular $\delta^{13}C$ value of acetic acid derived from pyruvate degradation was measured using HS-SPME-GC-Py-GC-C-IRMS [20]. The system consists of a first gas chromatograph, (Trace™ GC Ultra; Thermo Fisher Scientific Inc.) equipped with a capillary column (Nukol™, 30 m × 0.32 mm i.d., 1 μm film thickness; Supelco, PA, USA), connected to a second gas chromatograph (HP 6890 series; Hewlett-Packard Co., PA, USA) equipped with a second capillary column (HP-Plot Q 30 m × 0.32 mm i.d., 20 μm film thickness; Agilent Technologies Inc., CA, USA). Two gas chromatographs were connected through a pyrolysis furnace part (ceramic tube, 25 cm× 0.5 mm i.d.) operated at 1000 °C for pyrolysis of acetic acid. The pyrolytic products were separated using a second capillary column and were introduced into a combustion furnace (ceramic tube, 25 cm× 0.5 mm i.d., packed with CuO, NiO, and Pt wires) operated at 960°C. The second chromatograph was connected via Thermo GC

Isolink™ and Conflo-IV™ interfaces (both from Thermo Fisher Scientific Inc.) to a mass spectrometer (Finnigan Delta V™; Thermo Fisher Scientific Inc.). A transfer line between chromatographs was made using deactivated fused silica capillary (0.32 mm i.d.; GL Sciences Inc., Japan).

Acetic acid from pyruvate degradation was extracted using an SPME device, equipped with 85 μm thickness SPME fiber coated with carboxen/polydimethylsiloxane (Carboxen/PDMS stableflex™; Supelco, PA, USA). Extraction was conducted in a thermostatic chamber controlled to 25 °C: the non-stirred samples condition. The extraction time was 60 min. After HS-SPME extraction, the fiber was inserted into the injection port of the first gas chromatograph at 250 °C. Helium was used as a carrier gas for all experiments. Chromatographic conditions were the following: 2.0 mL/min flow rate of carrier gas and 10:1 split ratio. The first oven temperature program was the following: 100 °C (5 min), then rising to 190 °C (10 min) at the rate of 15 °C/min, and finally at 200 °C (2 min) at the rate of 15 °C/min. The second gas chromatograph was kept constantly at 40 °C.

3.2.4.2 Dual-inlet system

The dual-inlet system of an isotope ratio mass spectrometer (MAT 253™, Thermo Fisher Scientific Inc.) was used for the measurement of $\delta^{13}C$ value of CO_2 derived from pyruvate degradation.

3.2.4.3 Laser spectroscopy system

Bulk $\delta^{13}C$ values of sodium pyruvate (A, B, C, and D) were measured by laser spectroscopy system (Picarro Inc., CA, USA) consists of combustion module part, sample storage part (LIAISON, A0301) and Cavity ringdown spectrometer G1121-i part for isotopic CO_2 analysis.

3.3 RESULTS and DISCUSSION

3.3.1 Completeness of reaction and consistency of method

The experiment time that used for complete pyruvate decarboxylation was confirmed by experiment. The experiment of pyruvate decarboxylation by H_2O_2 was conducted respectively in ranges of 10, 30, 60, and 120 min. The acetic acid yield was measured using ion chromatography and was calculated using a calibration curve of the acetic acid standard. As shown in table 1, the yield of acetic acid reaches 99% at 60 min reaction time. At the 120 min range, it also had the same number around 99%, which implies that the reaction is completed at 60 min. For subsequent experiments, we used 60 min as the decarboxylation time.

The consistency of $\delta^{13}C_{C\text{-}1}$ ($\delta^{13}C$ value of C-1 of pyruvate) was confirmed by comparison of $\delta^{13}C_{CO2}$ between the value calculated using the mass balance equation of pyruvate (equation 2) and from direct measurement, which are expected to be the same. Table 2 shows that the differences of $\delta^{13}C_{CO2}$ values from the two methods were approximately ≤ 0.6‰, which is an acceptable range, showing that usage of the mass balance equation can obtain $\delta^{13}C_{CO2}$ value (equation 2). This consistency of method has also confirmed the acceptable use of HS-SPME-GC-Py-GC-C-IRMS, for which $\delta^{13}C$ of C-2 and C-3 ($\delta^{13}C$ value of C-2 and C-3 of pyruvate) are obtainable in a single step. Compare to previous

study[11,12], this study can process to the determination of intramolecular ^{13}C of pyruvate without the measurement of bulk $\delta^{13}C_{AcOH}$ value. From this, the $\delta^{13}C$ measurement of our method can reduce the unexpected errors occurs by duplicate sample preparation or switching between configuration systems [20], which benefits in both time and accuracy of analysis. $\delta^{13}C_{C\text{-}1}$ value can be calculated later using the mass balance equation of pyruvate.

3.3.2 Bulk and intramolecular $\delta^{13}C$ isotope distribution of sodium pyruvate

Details of $\delta^{13}C$ values of sodium pyruvate samples are presented in table 3-3. First, we obtained bulk $\delta^{13}C$ of sodium pyruvate, which are -22.6 ‰ (A), -22.6 ‰ (B), -21.3 ‰ (C), and -23.1 ‰ (D). For intramolecular $\delta^{13}C$ values, samples A, C, and D have $\delta^{13}C$ values in the pattern of C-3 > C-1 > C-2, whereas sample B has the $\delta^{13}C$ pattern of C-2 > C-3 > C-1. Figure 3-2 clarifies that we obtained intramolecular $\delta^{13}C$ distribution of pyruvate of two kinds. Moreover, same bulk $\delta^{13}C$ value of sample A and B have different patterns of intramolecular $\delta^{13}C$ values. These indicate that these pyruvates are potentially derived from different production processes or raw materials.

Pyruvate can be synthesized using chemical production, with tartaric acid and $KHSO_4$ as substrates (figure 3-3) [21]. Tartaric acid has two main pathways for chemical synthesis [22,23]. First is tartaric acid obtained from petroleum by-products, which inherit the $\delta^{13}C$ value from hydrocarbon substrate. Second is tartaric acid from cyanohydrin synthesis, which inherits the $\delta^{13}C$ value from the initial substrate (3 carbons from glyceraldehyde and 1 carbon from the cyano group). Recently, Zyakun et

al. determined the intramolecular $\delta^{13}C$ value in synthetic tartaric acid from chemical synthesis, finding ^{13}C depletion in its carboxyl carbon [23]. In general, without isotope fractionation, pyruvate is expected to inherit $\delta^{13}C$ value of the beginning tartaric acid. However, isotope fractionation can occur during actual production processes. Our hypothesis according to previous works is tartaric acid from chemical synthesis, which also has a similar trend of $\delta^{13}C$ values to acetic acid from chemical synthesis, which has depleted carboxyl carbon [8,23]. For intramolecular $\delta^{13}C$ value of pyruvate, the depletion of $\delta^{13}C$ value in carboxyl carbon (C-1) from C-2 and C-3 has been found in sample B. This trend is similar to the trend of intramolecular $\delta^{13}C$ values of acetic acid [8,24] and tartaric acid [23] from chemical synthesis. We might infer that sample B had high possibility to be produced by a chemical synthesis method, along with a good agreement to the $\delta^{13}C$ values of previous studies. However, without details of proprietary synthetic process of sample, the discussion about the exact pattern remains unclear. Further details related to isotope fractionation, which possibly occurred in production process, must be clarified in future works' discussion for concrete references.

Another pattern of intramolecular $\delta^{13}C$ values might have a different mode of production or substrate. Pyruvate can also be produced using biotechnological methods. Biotechnological methods have at least three methods: direct fermentation method, the resting cell method, and the enzymatic method [25]. The enzymatic method is simple, with a high conversion rate of the substrate. For example, lactate can be the substrate for pyruvate production using L-lactate catalyzed by glycolate oxidase in *Hansenula polymorpha* [26]. However, the high price of raw materials and some complicated processes for removal of by-products of production are shortcomings related to industrialize enzymatic methods for pyruvate

production. Consequently, direct fermentation and the resting cell methods have higher potential for mass production of pyruvate. Samples A, C, and D have found enrichment in $\delta^{13}C$ value in C-1 than C-2 and C-3, which also have the same trend of $\delta^{13}C$ values as those of biotechnological products reported from previous studies [8,23]. In this case, samples A, C, and D should have been regarded as products from biotechnological methods. Edens et al. [27] showed the intramolecular isotope effect data of pyruvate, associated with malic enzyme from chicken liver. Kinetic isotope effect numbers are determined for position C-1, C-2 and C-3 of pyruvate, which are 1.001, 0.987 and 1.021 respectively. This pattern leads to the ^{13}C-depletion at C-3 position while the C-2 position is the most ^{13}C-enriched in pyruvate. According to this pattern, pyruvate samples in this study did not match the exact pattern as previous studies. To make more concrete hypothesis, the intramolecular ^{13}C of pyruvate, associated with plant malic enzyme is needed. However, there are no references about isotope effect data associated with plant malic enzyme yet.

3.3.3 Bulk and intramolecular $\delta^{13}C$ isotope distribution of pyruvate supplement samples

Considering pyruvate supplement samples, we found their intramolecular ^{13}C distribution patterns to be similar to sample B, which is potentially, produced using chemical synthesis methods. However, if natural tartaric acid is the initial substance in chemical synthesis of pyruvate, then the intramolecular ^{13}C pattern might be different, according to the different pattern of $\delta^{13}C$ values of biogenic and abiogenic tartaric acid[23]. These intramolecular ^{13}C distributions of pyruvate can help us categorize the production process of pyruvate,

although further investigation of the intramolecular ^{13}C distribution pattern from plenty of natural samples and samples that are different from known processes must be done for additional explanations.

3.3.4 Bulk and intramolecular $\delta^{13}C$ isotope distribution of sodium pyruvate in previous study

We also considered the pattern of $\delta^{13}C$ values of the sodium pyruvate sample that used H_2O_2 degradation in a previous study [11], which has a similar pattern to that of sample B and which should fall into the category of chemical synthesis production, from the ^{13}C depletion in its carboxyl carbon than its C-2 and C-3.

3.4 Conclusion

Adoption of HS-SPME-GC-Py-GC-C-IRMS produces a more convenient analytical method for the determination of intramolecular $\delta^{13}C$ values in pyruvate. Pyruvate samples in this study have two patterns that are useful for categorizing samples into different production processes. Further studies of the natural pattern of the pyruvate from plants can be a good first step, followed by studies of pyruvate production by different known processes. These will help to distinguish the pyruvate samples into the correct categories of origin processes.

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Tables and Figures

Tables

Table 3-1. Yield percentage of the acetic acid by degradation time (n=3)

Degradation time (min.)	***Yield of acetic acid (%)***	***S.D. (n=3)***
10	76.4	0.4
30	86.2	0.9
60	99.1	0.5
120	99.9	0.0

Table 3-2. Mass balance calculation and direct measurement of $\delta^{13}C_{CO2}$ (C-1)

Sample (n=3)	***Calculation (‰)***	***Measurement (‰)***	***Difference (‰)***
A	-20.0 ± 0.7	-19.4 ± 0.1	0.6
B	-25.3 ± 0.6	-24.8 ± 0.3	0.5
C	-15.2 ± 0.7	-15.5 ± 0.0	0.3
D	-20.9 ± 0.1	-20.3 ± 0.3	0.6

Table 3-3. $\delta^{13}C$ measurement of sodium pyruvate samples and degraded fragments with $\delta^{13}C$ calculated from the mass balance equation and measurement values of $\delta^{13}C$

Sample	$\delta^{13}C_{C-1}$ (‰)	$\delta^{13}C_{C-2}$ (‰)	$\delta^{13}C_{C-3}$ (‰)	$\delta^{13}C_{AcOH}$ (‰)	Bulk $\delta^{13}C$ (‰)
A	**-20.0 ± 0.7**[a]	**-36.5 ± 0.3**	**-11.3 ± 0.4**	**-23.89 ± 0.5**	**-22.6 ± 0.2**
B	**-25.3 ± 0.6**	**-19.9 ± 0.2**	**-22.7 ± 0.3**	**-21.26 ± 0.4**	**-22.6 ± 0.2**
C	**-15.2 ± 0.7**	**-36.6 ± 0.4**	**-12.0 ± 0.5**	**-24.27 ± 0.6**	**-21.3 ± 0.2**
D	**-20.9 ± 0.1**	**-35.6 ± 0.5**	**-12.7 ± 0.5**	**-24.14 ± 0.7**	**-23.1 ± 0.0**
DP1	**-36.6 ± 0.8**	**-21.2 ± 0.3**	**-29.3 ± 0.1**	**-25.23 ± 0.3**	**-29.0 ± 1.5**
DP2	**-34.8 ± 1.5**	**-16.5 ± 0.3**	**-23.3 ± 0.3**	**-19.92 ± 0.4**	**-24.9 ± 2.7**
DP3	**-39.0 ± 1.5**	**-17.6 ± 0.0**	**-23.3 ± 0.3**	**-20.46 ± 0.3**	**-26.7 ± 2.6**
DP4	**-34.9 ± 0.3**	**-18.4 ± 0.2**	**-24.6 ± 0.2**	**-21.51 ± 0.3**	**-26.0 ± 0.7**
Sodium pyruvate using H_2O_2 oxidation (Melzer & Schmidt et al. (1987))	**-22.3**	**-19.6**	**-21.5**	**-20.57**	**-21.2**

[a]: standard deviation from the mean (*n*=3)

Figures

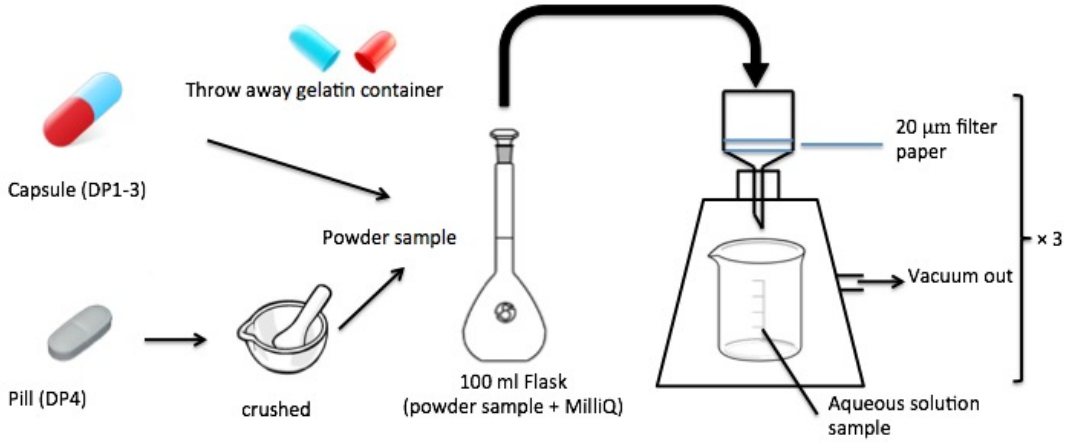
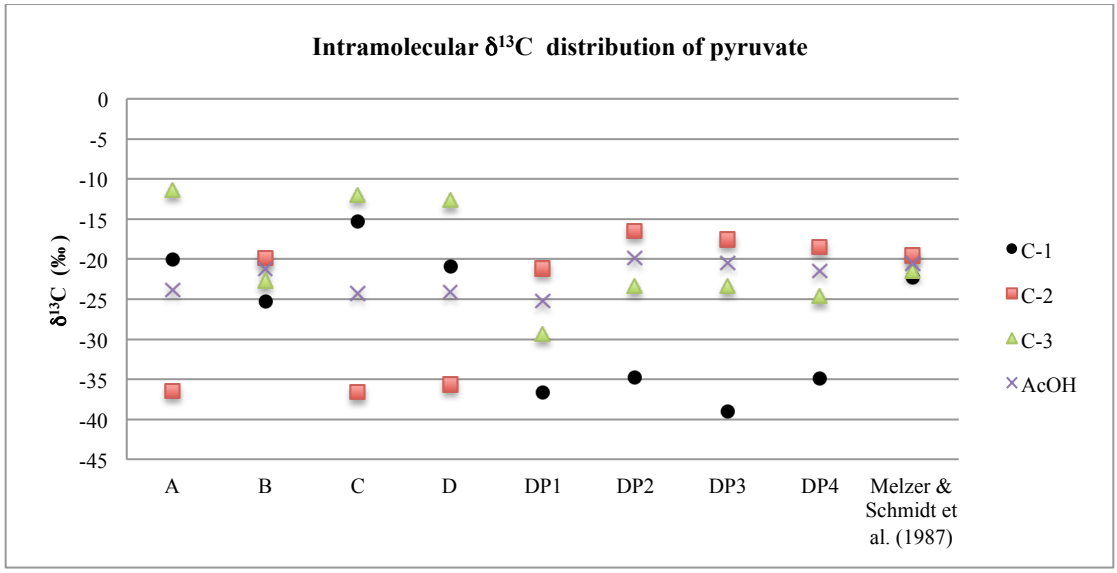
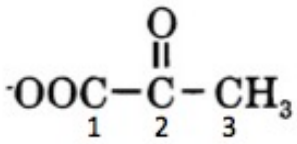


Figure 3-1. $\delta^{13}C$ patterns of commercial sodium pyruvate (A-D), pyruvate from diet supplement (DP1-DP4) and sodium pyruvate from previous study (Melzer and Schmidt, 1987).





Pyruvate carbon position

Figure 3-2. $\delta^{13}C$ patterns of commercial sodium pyruvate (A-D), pyruvate from diet supplement (DP1-DP4) and sodium pyruvate from previous study (Melzer and Schmidt, 1987).

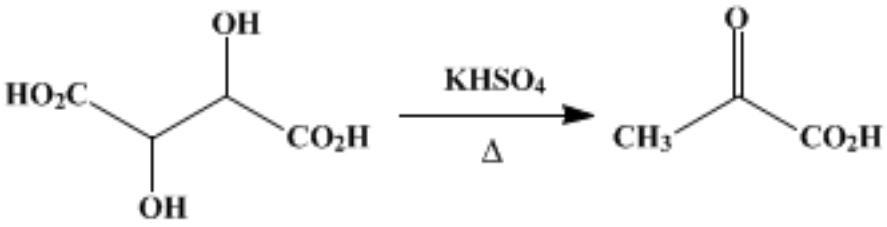


Figure 3-3. Chemical synthesis route of pyruvic acid from heating tartaric acid and $KHSO_4$.

Chapter 4

Conclusion and Future Prospect

4.1 Conclusion

This study has achieved in development of the position specific ^{13}C analytical method for organic acids, which consists of two parts. The first part is about the improvement of position specific ^{13}C analytical method of acetic acid. In previous study, solid phase micro extraction (SPME), gas chromatography/combustion-isotope ratio mass spectrometry (GC/C-IRMS) and gas chromatography/pyrolysis-gas chromatography/combustion-isotope ratio mass spectrometry (GC/Py-GC/C-IRMS) systems were used for the determination of intramolecular $\delta^{13}C$ of acetic acid[1]. Samples were extracted with SPME from headspace (HS) in vials and measured bulk and intramolecular $\delta^{13}C$ analysis respectively. This analytical method of acetic acid needed to switch between two configuration systems. Our improvement focuses on prevent this switching to avoid errors, which can occur from this change and duplication of sample preparation. Acetic acid lab standards[2] were used for making the calibration curve for intramolecular $\delta^{13}C$ calculation, which is derived from methyl (CH_3-) and carboxyl (-COOH) part. With this principle, the intramolecular and molecular $\delta^{13}C$ of acetic acid are able to obtain within single injection analysis. The HS-SPME-GC-Py-GC-C-IRMS method, which has successfully developed can be used for obtaining the $\delta^{13}C$ values of acetic acid for both intramolecular and molecular level in a single injection analysis. Having commercial vinegar as an application, intramolecular $\delta^{13}C$ distribution of samples was determined within 0.6‰ repeatability. By using this method, we can avoid the switching between two configuration systems and the duplication of sample preparation, which are factors for unexpected

errors. Also, the development of this method shows the importance of using intramolecular isotope standards for calibration.

The second part is about the improvement of position specific ^{13}C analytical method of pyruvate. Sodium pyruvate is the initial substance for experiment. First, bulk $\delta^{13}C$ of sodium pyruvate was measured by laser spectroscopy. In this study, sodium pyruvate has used H_2O_2 to decarboxylated into acetic acid and carbon dioxide, adapted from previous studies[3,4]. The completeness of the reaction is confirmed with the number of approximately 99.7% by the determination of manometric CO_2 with standard calibration curve. The bulk $\delta^{13}C$ of sodium pyruvate was obtained by laser spectroscopy within 0.3‰ repeatability. The intramolecular $\delta^{13}C$ of pyruvate consists of two parts, which are acetic acid and carbon dioxide respectively. The acetic acid part was determined by our developed HS-SPME-GC-Py-GC-C-IRMS method. The intramolecular $\delta^{13}C$ distribution of sodium pyruvate samples were obtained within 0.6‰ repeatability for both methyl and carboxyl part. Applying for pyruvate in diet supplementary as an application, the intramolecular $\delta^{13}C$ distributions of pyruvate in various samples were obtained within 0.3‰ repeatability for both methyl and carbonyl part. Later, $\delta^{13}C$ of carboxyl part can be calculated by using pyruvate mass balance equation or direct measurement of $\delta^{13}C$ using dual inlet system of IRMS system. In this study, two different $\delta^{13}C$ patterns were found in standard chemical and supplementary samples (figure 4-1). The successful improved method in this study can be a useful tool for the detection of production process and raw materials.

4.2 Perspectives for future study

The improvement of methodology in this study is proved to be a useful part for adaption and used in the development of analytical method of bigger molecule organic acids. The usage of developed method in this work showed the applicable usage in food authentication and quality control in industrial scale. In future study, this developed analytical method for determination of $\delta^{13}C$ has potential to apply for usage of other organic acids in pyruvate related pathways [5], for example, the three carbons organic acid (i.e. lactate) or bigger molecule organic acids (i.e. malate, citrate, oxaloacetate, etc…) those are able to be degraded by chemical reaction or specific enzymes, yield pyruvate as one of final products (figure 4-1).

The further development of analytical method is necessary for researches about the metabolic pathways those still have unclear information about carbon usage and isotope fractionation in some parts and still need more investigation. The carbon isotopes information of each organic acid will be the concrete references for people who study in this field. Important issues to be concerned for further development of methodology should aim to minimize the sample size and also overcome the hardship of extraction methods.

4.2.1 Attempt for natural sample study

4.2.1.1 Onion

Our first attempt to extract pyruvate from natural sample was focused on onion, which known for high amount of pyruvate[6]. In previous study by Ghandi et al. [6], 579.1 mg of pyruvate can be obtained by using 1 kg of onions, extracted by 95% of ethanol. In this study, 0.5 kg of onion was used for pyruvate extraction, which should be detected as

0.5 mmol pyruvate, based on calculation we used for sample preparation in previous chapters. We followed the extraction method and purification condition for onion's pyruvate of Ghandi et al. patent as a extraction model experiment. After the extraction was done, powder solution was diluted with 100 mL distilled water to make pyruvate aqueous solution. 0.2 of H_2O_2 was added to 1mL of pyruvate aqueous solution and reacted for 1 hour to obtain 0.5 mmol of acetic acid for subsequent analysis by GC-Py-GC-C-IRMS system.

However, acetic acid amount that had been detected after H_2O_2 degradation are lower than the detection limit as shown in figure 4-2 after using the same condition of experiment of chapter 2 and 3. The highest possibility of our fail attempt might depend on the losing of pyruvate during extraction method. In this case, we should think about the better way to obtain enough pyruvate from onion to match the limit of detection of device. Otherwise, we might change to more suitable instruments or the way to analyse the $\delta^{13}C$ of pyruvate from natural by the specific instruments instead.

4.2.1.2 Beer

We try our second attempt of pyruvate extraction on beer. For pyruvate extraction and purification, solid phase extraction Strata®Sax (55 µm, 70 Å) 200 mg, 3 mL (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) was used. The extraction method and sequence is as same as Rodrigues et al. [7]. Concentration of pyruvate had been determined by using Agilent 1260 Infinity high-pressure liquid chromatography (HPLC) system (Agilent Technologies, CA, USA) after extraction. The calibration curve of pyruvate standard that varied the concentration range from 0.01-100mM (figure 4-3) was made. HPLC was

operated under nebulizer temperature at 80°C and evaporator temperature at 70°C with evaporator gas flow at 1.60 standard liter per minute (SLM). The wavelength detection was set at 220 nm with response time > 0.1 minute (2 second). Acetonitrile plus and 20mmol/l phosphate buffer were used as mobile phases in this experiment. COSMOSIL HILIC, 10mm I.D. × 250 mm packed column (Nacalai Tesque, Inc, CA, USA) was used as stationary phase. The Sample injection volume was 50 μl with the flow rate at 2 mL/min.

Pyruvate had been detected after the extraction by using the Solid Phase Extraction (SPE) device along with the adaption of the method from previous works [7,8]. The concentration is approximately 0.05-0.1 mM as shown in figure 4-4. In this case, pyruvate had too low concentration for subsequent analysis of $\delta^{13}C$ in beer samples. The possibilities of our failed attempt might cause by the very low amount of pyruvate in beer, the insufficient HPLC column usage or the unsuitable detecting device. In future studies, important things those need to be improved are the suitable extraction and instruments for pyruvate analysis. Then the suitable process will lead to the attempt of pyruvate extraction from various types of samples as much as possible, in order to build the strong evidences those can confirm the ranges and patterns of carbon isotope compositions of pyruvate. After that the extension of studies to other organic acids can be the next things, which will help us clarify the unknown things in metabolic pathways of organic acids.

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Tables and Figures

Figures

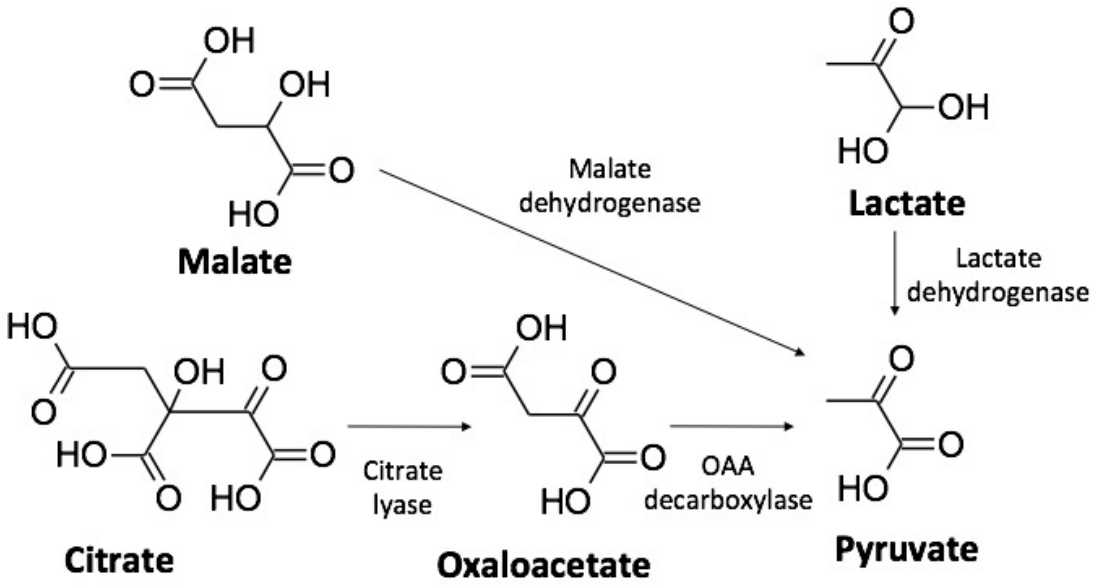

Figure 4-1. Metabolic pathways of pyruvate related organic acids which have potential for potential next development of methodologies.

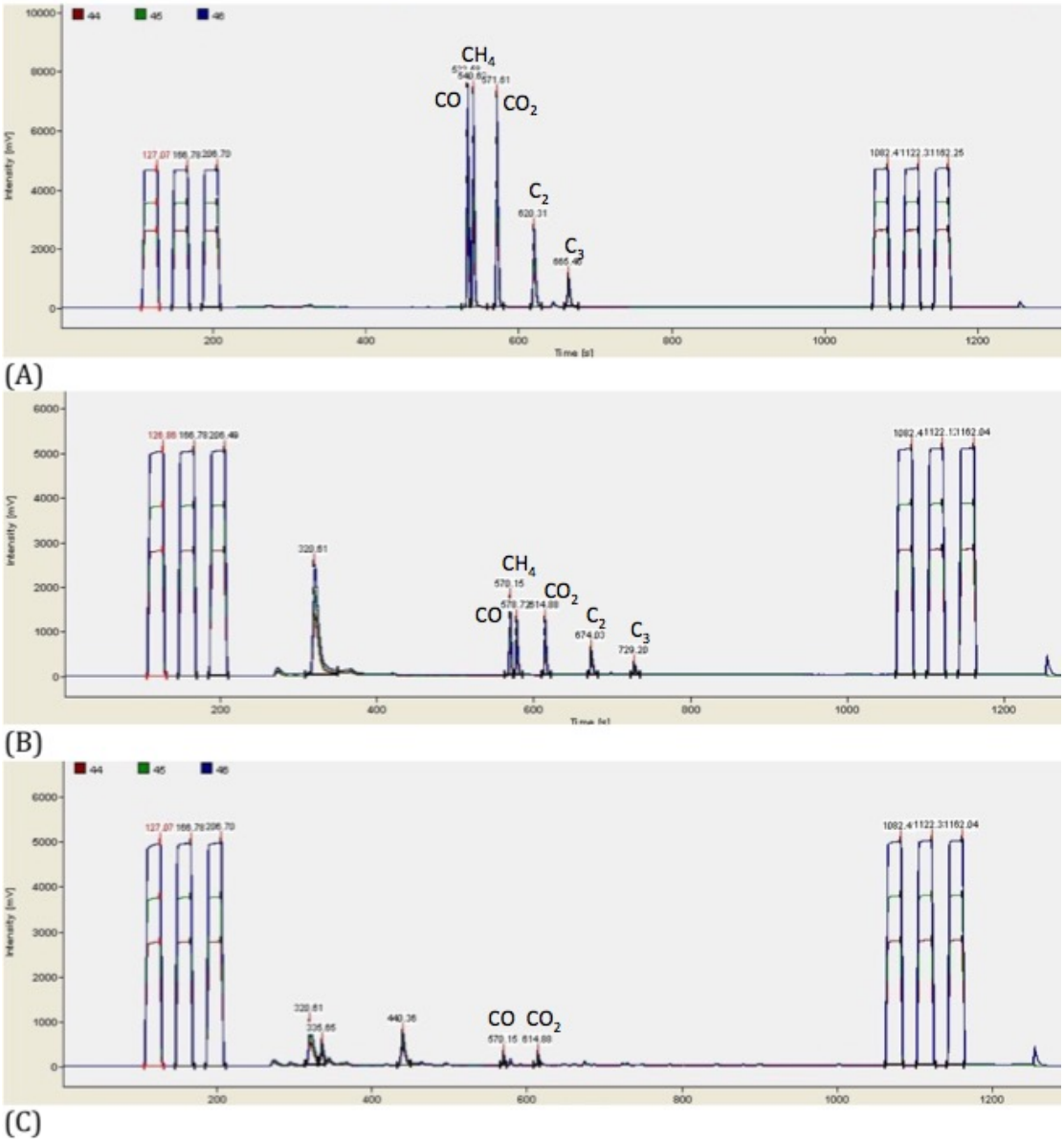

Figure 4-2. Chromatogram of acetic acid of different samples: (A) pure acetic standard, (B) acetic acid from H_2O_2 degradation of pyruvate in pyruvate supplement and (C) acetic acid from H_2O_2 degradation of pyruvate in onion.

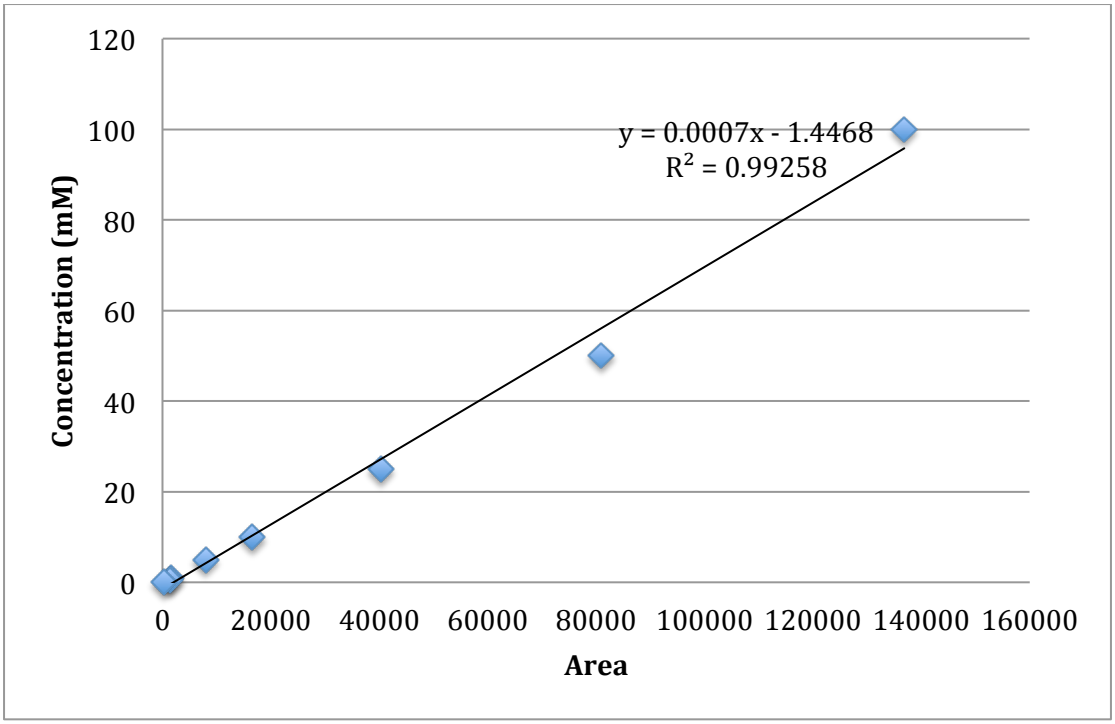

Figure 4-3. Calibration curve of pyruvate by concentration.

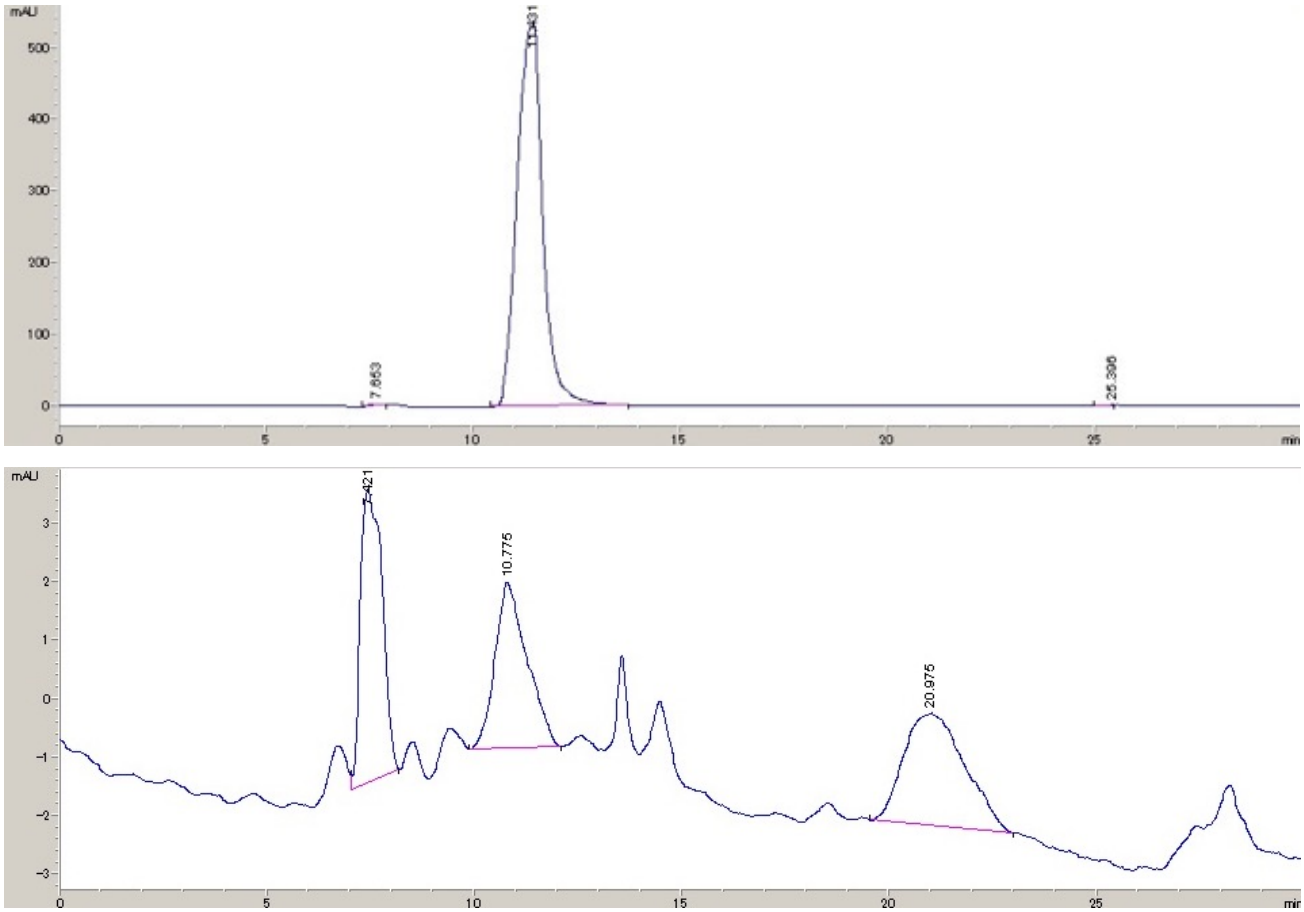

Figure 4-4. Chromatogram from high-pressure liquid chromatography (HPLC): (above) pyruvate aqueous solution 10 mM, (below) beer sample.

Publication List

1. T. Nimmanwudipong, K. Yamada, A. Gilbert, N. Yoshida (2015) Analytical method for simultaneous determination of bulk and intramolecular ^{13}C-isotope compositions of acetic acid. *Rapid. Commun. Mass Spectrom.* **29**, 2337–2340
2. T. Nimmanwudipong, N. Zhang, K. Yamada, A. Gilbert, N. Yoshida (2015) Determination of Intramolecular ^{13}C Isotope Distribution of Pyruvate by Headspace Solid Phase Microextraction-Gas Chromatography-Pyrolysis-Gas Chromatography-Combustion- Isotope Ratio Mass Spectrometry (HS-SPME-GC-Py-GC-C-IRMS) Method. *J. Anal. Bioanal. Tech.* **7**, 293

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