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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

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Abstract

This paper presents the improvement of analytical methods for intramolecular carbon isotope compositions ($\delta^{13}C$) of pyruvate. Decarboxylated by H_2O_2, pyruvate yields acetic acid and CO_2. Headspace solid phase micro-extraction-gas chromatography-pyrolysis-gas chromatography-combustion-isotope ratio mass spectrometry (HS-SPME-GC-Py-GC-C-IRMS) was used to measure the intramolecular $\delta^{13}C$ values of acetic acid. $\delta^{13}C$ value of CO_2 can be later calculated using mass balance equation. The method's consistency was confirmed by comparison of the $\delta^{13}C$ value of CO_2 from calculation to its direct measurement. Results of this study confirmed the method improvement because pyruvate ^{13}C intramolecular distribution patterns were obtained. Two intramolecular ^{13}C distribution patterns for commercial chemical reagents were found using this developed method. Intramolecular ^{13}C distribution patterns for pyruvate were found for application in dietary supplements. Its origin was inferred. The method presented herein is expected to be a useful tool for categorization of pyruvate into different intramolecular ^{13}C distribution patterns, which might indicate different production processes or raw materials.

Keywords: Pyruvate; Dietary supplement; Intramolecular isotope distribution; Food authenticity; Quality control

Introduction

Isotope analysis has been applied to facilitate identification of the origin, metabolic pathways, and biosphere-atmosphere interaction of organic materials [1-4]. Compound Specific Isotope Analysis (CSIA) is typically used to ascertain the isotopic composition of a target compound. However, in some cases, CSIA data alone are not sufficient for identification of the compound's origin. Position-Specific Isotope Analysis (PSIA) has provided information related to the heterogeneous isotope distribution of organic compounds including amino acids, acetic acid, fatty acids, sugars, ethanol, and hydrocarbons [5-9]. This information is crucially important for the investigation of synthetic processes and metabolic pathways of the target compound. These isotope analysis techniques are also applied in the food industry for quality control [1,2].

Pyruvate, a key metabolite for carbohydrate metabolism, is necessary to trigger a plant's citric acid cycle, fat, and protein metabolism. It can also be useful as a dietary supplement to increase the metabolic rate [10]. Pyruvate influences the isotopic contents of respired CO_2 and its related metabolites. Therefore, its isotope signature would be beneficial for studying the authenticity and metabolic pathways in plants.

The main objective of this study is to improve the analytical method for intramolecular ^{13}C distribution of pyruvate, which can be degraded into acetic acid and CO_2 using H_2O_2 [11,12]. Results confirm the success of this technique: we obtained a pyruvate ^{13}C intramolecular distribution pattern.

Additionally, we applied this method to ascertain the intramolecular ^{13}C distribution in a pyruvate sample from pyruvate supplement pills. Dietary supplements are convenient choices that provide essential nutrients. Because of their high demand, the number of manufacturers has increased rapidly in the past few years. One way to remain competitive in the market is to decrease manufacturing costs to the greatest extent possible. Commonly, people prefer authentic products from natural sources. However, the same commercial synthetic substance, which is obtainable by the derivatization of petroleum or coal, offers a rapidly producible and cheaper alternative than the natural extract from biogenic sources [13-15]. According to the Food and Drug Administration (FDA) in some countries, synthetic substances are illegal: their manufacture is prohibited [16]. To minimize the risk of illegality, a method is needed to help differentiate between synthetic and natural substances [17-19]. This study considered the potential origin of pyruvate in dietary supplement based on the intramolecular ^{13}C distribution.

Materials and Methods

Annotations

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).

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

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

$$\delta^{13}C_{Pyruvate} = [2(\delta^{13}C_{AcOH}) + \delta^{13}C_{CO_2}] / 3 \quad (2)$$

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

$$\delta^{13}C_{AcOH} = (\delta^{13}C_{CH_3} + \delta^{13}C_{COOH}) / 2 \quad (3)$$

Where $\delta^{13}C_{CH3}$ value and $\delta^{13}C_{COOH}$ value respectively represents 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).

Chemicals

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. 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. 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.

Degradation of pyruvate

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

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

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. Regarding 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.

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.

Carbon isotopic analysis

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 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.

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. Bulk $\delta^{13}C$ values of sodium pyruvate (A, B, C, and D) were measured using cavity ring-down laser spectroscopy (Picarro G1121i; Picarro Inc., CA, USA).

Results and Discussion

Completeness of reaction and consistency of method

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 $\delta^{13}C_{C-1}$ ($\delta^{13}C$ 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. Without the measurement of bulk $\delta^{13}C_{AcOH}$ value, the $\delta^{13}C$ measurement can reduce the unexpected errors occurs by duplicate sample preparation or switching between configuration systems [20]. $\delta^{13}C_{C-1}$ value can be calculated later using the mass balance equation of pyruvate.

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. 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 1 clarifies that we obtained intramolecular $\delta^{13}C$ distribution of pyruvate of two kinds. Moreover, same bulk $\delta^{13}C$ value of A and B samples 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 [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 potential 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 biological products reported from previous studies [8,23]. In this case, samples A, C, and D should have been regarded as products from biotechnological methods. 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.

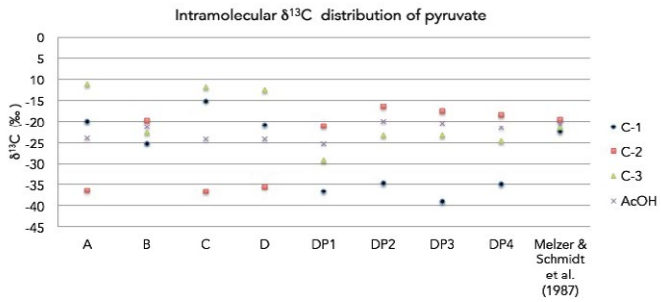

Figure 1: $\delta^{13}C$ pattern of commercial sodium pyruvate (A-D), pyruvate from diet supplement (DP1-DP4) and sodium pyruvate from a previous study [11].

Degradation time (min.)	Yield of acetic acid (%)
10	76.4
30	86.2
60	99.1
120	99.9

Table 1: Yield percentage of acetic acid by degradation time.

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

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 ± 104	-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 degradation [11]	-22.3	-19.6	-21.5	-20.57	-21.2

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

Table 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 $\delta^{13}C$.

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.

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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