Extraction of Isovitexin from Melinjo *(Gnetum Gnemon L.)* Leaves Using Mixtures of Liquid Carbon Dioxide and Ethanol

Yusraini Dian Inayati Siregar[1, 6], Kenji Mishima[1,2,*], Ryo Kawakami[1], Shota Ito[1], Yuuta Inoue[1], Tetsuya Hirota[1], Tanjina Sharmin[1], Takafumi Kato[1],Takunori Harada[3], Makoto Misumi[2,4], Hideaki Orii[2], Tadashi Suetsugu[2,4], Keiichi Irie[5], Kenichi Mishima[5], Kumiko Sakai[6], Kenji Sakai[7], Hirofumi Kawamura[8], Hilyatuz Zahroh[1,9], Nurelela[9], Adi Riyadahi[9], Lily Surayya Eka Putri[9] and Agus Salim[9]

[1]Department of Chemical Engineering, Faculty of Engineering, Fukuoka University, 8-19-1, Nanakuma Jonan-ku, Fukuoka 814-0180, Japan

[2]Academia, Industry, Government Collaborative Research Institute of Composite Material, Fukuoka University, 8-19-1, Nanakuma Jonan-ku, Fukuoka 814-0180, Japan

[3]Department of Applied Chemistry, Faculty of Engineering, Oita University, 700, Dannoharu, Oita, Oita 870-1124, Japan

[4]Department of Electronics Engineering and Computer Science, Faculty of Engineering, Fukuoka University, 8-19-1, Nanakuma Jonan-ku, Fukuoka 814-0180, Japan

[5]Department of Neuropharmacology, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1, Nanakuma Jonanku, Fukuoka 814-0180, Japan

[6]Research Promotion Institute, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasama-machi, Yufu-Shi, Oita, 879-5593, Japan

[7]Department of Bioscience and Biotechnology, Graduate School of Bioresources and Bioenvironmental Sciences, Kyushu University, Fukuoka 812-8581, Japan

[8]Department of Seasoning & Foods Division, San-Ei Gen F.F.I., Inc., 1-1-11, Sanwa-cho, Toyonaka, Osaka 561-8588, Japan

[9]Faculty of Science and Technology, Syarif Hidayatullah State Islamic University (UIN) Jakarta, JL.Ir.H.Juanda Ciputat, Tangerang, 15412, Indonesia

Abstract

Isovitexin from Melinjo *(Gnetum gnemon L.)* leaves was extracted using mixtures of liquid CO_2 and ethanol. For finding out the optimum extraction condition, extraction tests were performed at 5, 20 and 25 °C under pressures ranging from 8 to 14 MPa. A conventional extraction by ethanol was performed to compare the extraction yield of isovitexin. We conducted qualitative and quantitative analyses for isovitexin in the extract by using HPLC. The effects of three operating parameters, including temperature, pressure and the mole fraction of ethanol in liquid CO_2, on the extraction yield were investigated using the single-factor method. The yield of isovitexin in the extract was significantly improved by adjusting the extraction temperature and the solvent composition.

1. Introduction

Melinjo *(Gnetum gnemon L.)* is an indigenous plant that is widely distributed in Indonesia and has been traditionally used only as an ingredient for sour vegetables or as a snack. The leaves of *Gnetum gnemon* have thick-walled cellulosic fibers, which provide healthy nutrition [1], including cyclopropene fatty acids [2]. Moreover, a number of flavones extracted from melinjo exhibit pharmacological activities, such as antioxidant properties. Isovitexin (Figure 1) is one of the common flavones found in the extracts of the leaves of *Gnetum gnemon*, which has broad applications and is an important material for the food, pharmaceutical and chemical industries.

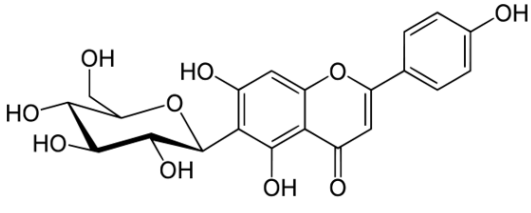

Figure 1 Chemical structure of isovitexin

*Corresponding author.
E-mail address : mishima@fukuoka-u.ac.jp (K. Mishima), Tel: +81-92-871-6631 Ext. 6428; Fax: +81-92-865-6031

In 2010, Santoso et al. reported on the antioxidant components and activities of the edible parts of *Gnetum gnemon* (young and mature leaves) [3], and an evaluation of the *in vitro* antioxidant activity of the leaves of *Gnetum gnemon* has also been described [4]. Additionally, in 2011, Wazir, Ahmad, Muse, Mahmood and Shukor [5] reported the antioxidant activities of different parts of *Gnetum gnemon* (leaf, bark, twig, and seeds). These findings make isovitexin attractive to an expanding international market to use as a sedative and anxiolytic [6], an antidiabetic substance [7], and as an alpha-glucosidase inhibitor [8]. Therefore, an increasing demand for food antioxidants from natural sources has encouraged the extraction of isovitexin from the leaves of *Gnetum gnemon*.

Many papers have reported various extraction and separation methods for flavones from several types of leaves. The flavones are generally extracted with organic solvents, such as ethanol, methanol, acetone, and toluene. In the past several years, supercritical carbon dioxide (SC-CO_2) extraction has received considerable interest as a separation technique in many fields. In our previous papers [9] - [11], the possibility of extraction and separation of flavonoids using SC-CO_2 was demonstrated, and the solubility data of flavonoids in SC-CO_2 were accumulated. The use of SC-CO_2 for the microcoating of flavonoids for drug delivery systems was also reported [12]. The feasibility of using the SC-CO_2 process in the industry was discussed elsewhere [13] - [15]. In this study, we used liquid CO_2 as a solvent because CO_2 is a relatively benign solvent and will not be produced in concentrations that would substantially impact global environmental atmospheric levels, and CO_2 is non-flammable, inexpensive and, more importantly, relatively non-toxic. In spite of these advantages, little has been reported regarding the extraction of flavones from leaves using SC-CO_2 on an industrial scale. SC-CO_2 is not widely used because the excess sensitivity of the solvent and the low solubility of flavones in SC-CO_2. The density change of SC-CO_2 is easily induced with a change in the operating pressure. As previously reported, to overcome the disadvantage of the excess sensitivity of the solvent power of the SC-CO_2, we proposed to replace SC-CO_2 with liquid CO_2 as the extraction solvent [11]. When the gas phase and the liquid phase coexist in an extraction vessel, the change in the density of liquid CO_2 is very small upon changing the operating pressure. As a result, in the liquid CO_2 extraction process, the excess sensitivity of solvent power with a change in the operating pressure can be protected.

In this work, we experimentally demonstrated the extraction of isovitexin from leaves of *Gnetum gnemon* using an extraction solvent of liquid CO_2 and ethanol added as a cosolvent. In addition, the effects of the operating conditions, such as temperature, pressure, and the mole fraction of ethanol in a liquid CO_2 solution, on the yield of the extracted isovitexin (mass of extract) were determined using the single-factor method.

2 Experimental

2.1 Reagents

The leaves of *Gnetum gnemon* were purchased from local markets in Indonesia. Isovitexin was purchased from Wako Co. Ltd, Osaka, Japan. Methanol, ethanol and acetic acid were purchased from Wako Co. Ltd. Analytical grade methanol and ethanol were used as the solvents for the conventional liquid extraction process and as solvents in the HPLC analysis. High-purity CO_2 (over 99%, Fukuoka Sanso Co. Ltd., Fukuoka, Japan) was used as received.

2.2 Extraction Procedures Using Mixtures of Liquid CO_2 and Ethanol

The leaves of *Gnetum gnemon*, which were dried in the shade for 5 days, were ground and powdered with a freezer mill 6770 (SPEX CentriPrep Co. Ltd. , New Jersey, USA) as described before [16]. The average particle diameter was less than 1 mm. The samples were stored in a cold and dark place because of the photosensitivity of the samples. The *Gnetum gnemon* leaf powders (0.1 g) were placed in a high-pressure cell (an extraction cell) with a liquid CO_2 extraction apparatus (SFC; super200, JASCO Co. Ltd., Tokyo, Japan). More detailed descriptions of the apparatus and the operating procedures were given in our previous papers [11] - [17]. A high-pressure cell (Akico Co., Tokyo, Japan, SCV50A), approximately 50 cm^3 in volume, was used. The system pressure was controlled by means of a back-pressure regulator (880-81, JASCO, accurate to 0.1 MPa) and monitored by a digital pressure gauge (Shinwa Electronics Co., Tokyo, Japan, model DD-501, accuracy ± 0.3%). The temperature was controlled within ± 0.1 °C using a water bath. The powders of *Gnetum gnemon* leaves (0.10 g) and a required amount of ethanol were placed in the high-pressure extraction cell for the pure liquid CO_2 extraction and the extraction using a mixture of liquid CO_2 and ethanol. The solvent composition x_{EtOH} was defined by the mole fraction of ethanol in the mixture of liquid CO_2 and ethanol. The mole fraction of ethanol, x_{EtOH}, in the feed composition to be prepared was determined by adjusting the amount of ethanol. Liquid CO_2 was supplied from a gas cylinder at a constant flow rate (2 cm^3/min) into the extraction cell via an HPLC pump (SCF-get, JASCO, Tokyo, Japan), and the pump-head was cooled by a cooling unit to ensure the CO_2 remained in its liquid state. The

liquid CO_2 was pumped into the high-pressure cell through a stainless steel pre-heater tube (length: 3 m, diameter: 1/8 in). The densities of the liquid CO_2 at 5, 20 and 25 °C under 10 MPa pressure were 945, 849, and 812 kg/m^3, respectively. The pressure of the extraction cell was controlled by adjusting the backpressure regulator (880-81, JASCO). The backpressure regulator valve was warmed to avoid CO_2 solidification caused by the Joule–Thomson effect. Occasionally, the effluent from the extraction cell was collected in a sample trap (i.e., ethanol). After the desired time had elapsed, the residue in the tubes was washed with liquid CO_2 and collected in another sample trap.

The extraction process was mainly affected by the solubility of the solute. The solute solubility was sensitive to the temperature, solvent composition, and density of the solvent mixture of CO_2 and ethanol. In our study, the effects of the operating temperature, pressure, and solvent composition were studied to optimize the extraction yield. The time for extraction was set at 60 min. We accounted for the critical temperature (*Tc*=31 °C) and pressure (*Pc*=7.8 MPa) of CO_2 and chose the extraction conditions to avoid the supercritical condition, which is too sensitive for reliable operation. The extraction was performed at temperatures of 5, 20 and 25 °C, and pressures of 8, 10 and 14 MPa. After extraction, the extracts were collected from an expansion valve using a U-tube ethanol trap, as well as from all of the tubes that were located downstream from the extraction cell and from the inside of the extraction cell at atmospheric pressure. The extracts remaining in the high-pressure cell were also collected and separated from the *Gnetum gnemon* leaf powder via filtration with ethanol. The compositions of the collected samples were analyzed using HPLC (high-performance liquid chromatography).

2.3 Conventional Liquid Alcohol Extraction Procedures

To compare the possibility of liquid CO_2 extraction and conventional alcohol (methanol or ethanol) extraction more rationally, 0.1 g of powders from the leaves of *Gnetum gnemon* was extracted with a solvent (methanol or ethanol; 50 cm^3 in volume). Extraction was performed using a water bath (Eyela SB-24, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) with a shaker (Eyela SS-8) and a heating circulator (Eyela T-80) which set the temperature at 25 °C controlled with an accuracy of 0.1 °C. Sampling was performed at 10, 20, 30, 60, and 120 min after starting the extraction.

2.4 HPLC Analysis

The HPLC system consisted of a Tosoh LC-8010 system equipped with a UV detector. The detection wavelength was set at 343 nm. Separation of the extract was performed using a TSK-GEL (Tosoh Co., Shunan city, Japan) ODS-80Ts column (1504.6 mm) set at 40 °C. The injection volume was 100 µL. Two mobile phases were used during the separations: (A) HPLC grade methanol and (B) 0.1% acetic acid in water. The methanol gradient profile is reported in Table 1. The flow rate of the solvent was set at 1.0 mL/min.

Table 1 Time course of methanol gradient for HPLC analysis

Time [min]	0	25	40	50	60	65	75
Methanol [vol%]	5	40	60	90	90	5	5

3 Results and Discussion

The HPLC profiles reported by Cristea et al. [18] were modified to more accurately separate the components in the extraction medium. The validity of this varying mobile phase composition was confirmed by the results of the composition analyses of the liquid CO_2 extract and the standard samples of isovitexin. The chromatograms of the isovitexin standard and the extract of *Gnetum gnemon* leaves via pure liquid CO_2 extraction at 25 °C and 10 MPa are shown in Figure 2. Based on the analysis of the standard isovitexin sample, we confirmed that the peak appearing at 32.72 min was isovitexin.

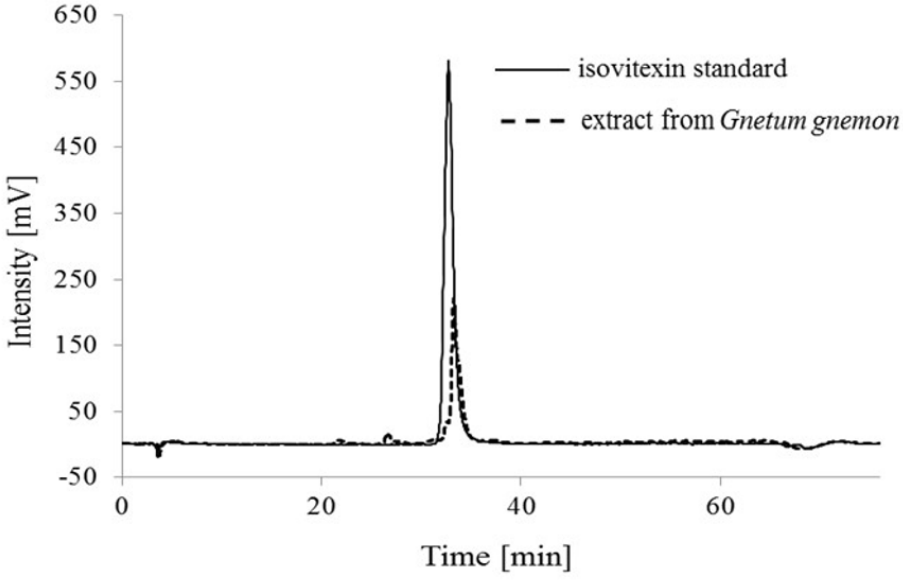

Figure 2 HPLC chromatogram of the isovitexin standard and the compounds extracted from *Gnetum gnemon* leaves using the mixture of liquid CO_2 and ethanol (x_{EtOH} = 0.131) at 25 °C and 10 MPa

To characterize the solvent extraction behavior of isovitexin from the leaves of Melinjo *(Gnetum gnemon L.)* a time-dependent assay of extraction was performed using methanol or ethanol as the solvent at 25 °C (Figure 3).

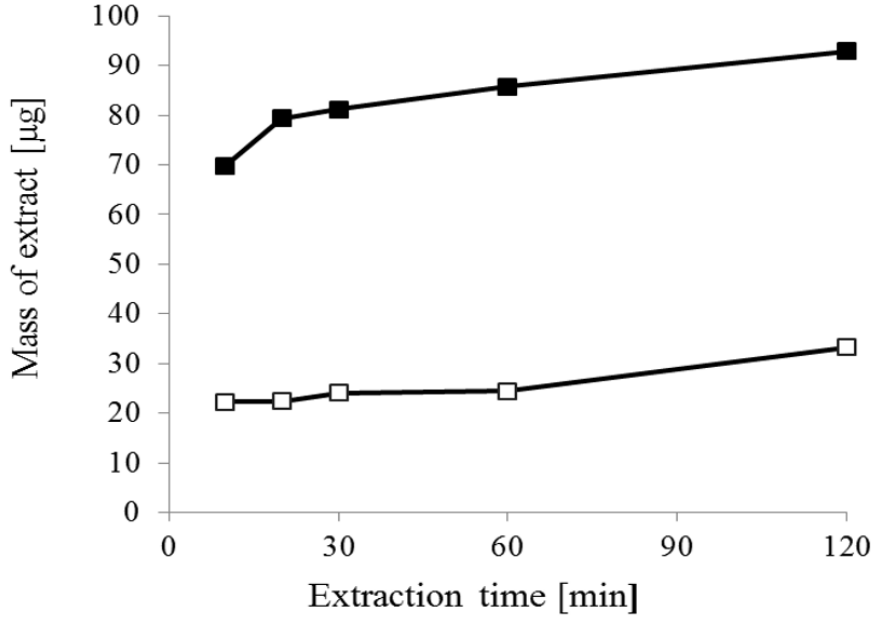

Figure 3 The yield of isovitexin extracted from 0.1 g powder of the leaves of *Gnetum gnemon* using methanol (■) and ethanol (□) as the solvent at 25 °C under atmospheric pressure at different time points

As described previously [11], the extraction was performed using a heating and shaking apparatus, and sampling was performed at several time points. Qualitative and quantitative information was obtained via HPLC. The mass of isovitexin extract generally increased over 0-60 min before reaching saturation. Therefore, the appropriate time of treatment was set at approximately 60 min. The maximum mass of isovitexin extract extracted from 0.1 g of *Gnetum gnemon* leaves with ethanol or methanol was 24.4 μg and 85.7 μg, respectively. From this result, we confirmed that the solvent power of ethanol was lower than that of methanol. Although the mass of isovitexin extract extracted using methanol exhibited maximum yield, methanol remaining in the extract must be removed because methanol's toxicity. Therefore, ethanol was further used as cosolvent due to minimal toxicity.

To study the effect of temperature on the total amount of isovitexin extracted using mixtures of liquid CO_2 and ethanol as a cosolvent (x_{EtOH}=0.131), the temperature of the water bath was set to 5, 20 and 25 °C. For the calculation of the liquid CO_2 density at various temperatures and pressures, we used the density data of CO_2 [18]. The pressure was set at 10 MPa, and the extraction was performed for 60 min. The experiment was repeated 3 to 6 times. The temperature of the air bath was set to 5, 20 and 25 °C. Changing the temperature significantly affects the density of liquid CO_2, to balance mole fraction ratio of ethanol to liquid CO_2 (x_{EtOH}) in the cell additional ethanol was added to the extraction cell as a cosolvent before the extraction.

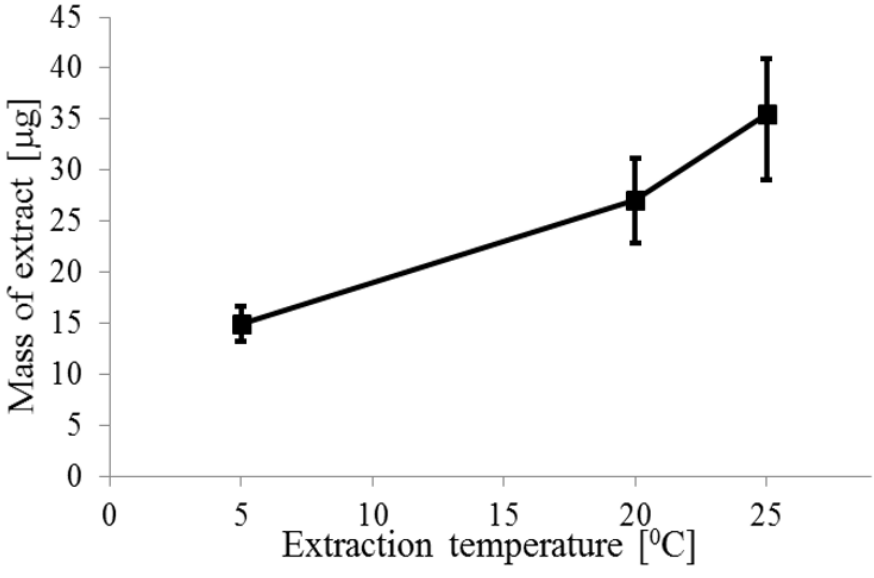

Figure 4 Effect of the extraction temperature on the mass extracted from the leaves of *Gnetum gnemon* (0.1 g) by the mixture of liquid CO_2 and ethanol (xEtOH = 0.131) at 10 MPa. Extraction was performed for 60 min

Figure 4 shows the effect of temperature on the yields of isovitexin. In general, the extraction yield gradually increased by 25.4% with increasing temperature. The highest yield of isovitexin was obtained at 25 °C. This behavior was consistent with the results of other liquid CO_2 extractions [11, 16, 17]. The densities of the liquid CO_2 at 5, 20 and 25 °C at 10 MPa were 945, 849, and 812 kg/m^3, respectively. Under these experimental conditions, changes in fluidity and diffusivity of the mixture of CO_2 and ethanol at each temperature should be considered in the vicinity of the critical point, the solvent molecular activity induced by temperature was considered to be more predominant than that induced by the solvent density. In additions, we should take into account the high-density gas phase, which existed above the liquid phase of CO_2 and was too sensitive in terms of pressure for reliable operation.

Finally, to clarify the effect of the mole fraction of ethanol over liquid CO_2 (x_{EtOH}) on the yield of isovitexin, we performed the extraction using ethanol and liquid CO_2 alone and in combination; x_{EtOH} = 0; 1 denoted pure CO_2 and ethanol, respectively, as shown in Figure 5. The pressure and temperature were set to 10 MPa and 25 °C, respectively, and the extraction was performed for 60 min. To change the mole fraction of ethanol delivered to mixtures of liquid CO_2 and ethanol (x_{EtOH}) over the range of 0 to 1, we set three experimental schemes: x_{EtOH} = 0 only liquid CO_2, and x_{EtOH} = 1

was the traditional extraction with ethanol without liquid CO_2. For the other ranges of x_{EtOH}, the extraction was performed with mixtures of liquid CO_2 and ethanol with the ethanol mole fractions set to 0.131, 0.26 and 0.39, which revealed the effects of the mole fraction of ethanol more precisely. The maximum yield of isovitexin, as shown in Figure 5, was obtained at x_{EtOH} = 0.131 as expected. This result implied that lower or higher mole fractions of ethanol reduce the yield of isovitexin under our extraction conditions. As expected, this result was consistent with the results of our previous CO_2 extraction of xanthones and resveratrol, as well [16, 17].

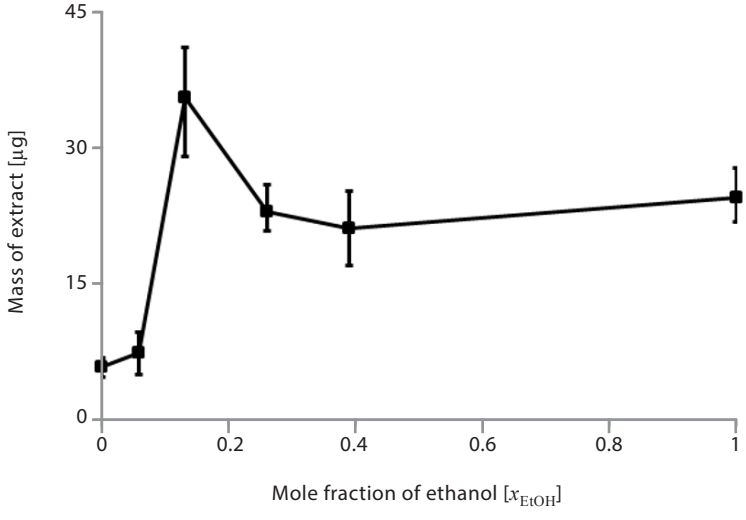

Figure 5 Effect of the mole fraction of ethanol in mixtures of liquid CO_2 and ethanol on the mass of isovitexin extracted from 0.1 g of of *Gnetum gnemon* leaves at 25 °C and 10 MPa

A comparison of the mass of isovitexin extracted from the leaves of *Gnetum gnemon* (0.10 g) using ethanol at 25 °C under atmospheric pressure, and pure liquid CO_2 and a mixture of CO_2 and ethanol (ethanol mole fraction in solvent, x_{EtOH}=0.131) at 25 °C and 10 MPa is shown in Figure 6.

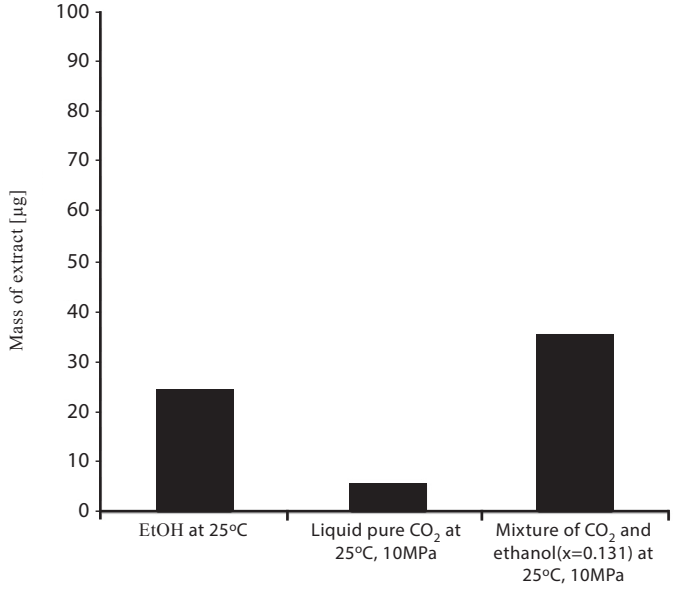

Figure 6 Comparison of the mass of isovitexin extracted from the leaves of *Gnetum gnemon* (0.1 g) using ethanol at 25 °C under atmospheric pressure, and pure liquid CO_2 and the mixture of liquid CO_2 and ethanol (ethanol mole fraction, x_{EtOH}=0.131) at 25 °C and 10 MPa

Extraction was performed for 60 min. Although CO_2 has an advantage to be biologically benign and easy to separate from an extract, CO_2 showed a disadvantage of low solvent power to extract isovitexin (the mass of extract is 5.8 µg) because of its non-polarity. On the other hand, the mass of the isovitexin extract obtained using ethanol was 24.4 µg. However, this figure clearly showed the highest yields of isovitexin were enhanced using the mixture of CO_2 and ethanol (the ethanol mole fraction in solvent, x_{EtOH}=0.131). These results implied that the mixture of CO_2 and ethanol (the ethanol mole fraction in solvent, in solvent, x_{EtOH}=0.131) had high affinity and suitable polarizability for isovitexin. Therefore, ethanol was considered to be a good cosolvent for the liquid CO_2 extraction of isovitexin.

4 Conclusion

Isovitexin was extracted from the leaves of *Gnetum gnemon* using mixtures of liquid CO_2 and ethanol as the liquid solvent and a co-solvent, respectively. The extraction temperature and solvent composition exhibited a large impact on the yield of isovitexin. The optimal condition was found at an extraction temperature of 25 °C, extraction time of 60 min and ethanol mole fraction of 0.131. Using these parameters, the average yield of isovitexin was improved to 35.5 µg from 0.1 g of *Gnetum gnemon* leaves.

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