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RESEARCH ARTICLE

CHANGES IN AMYGDALIN CONTENT OFTHE JUICE FROM JAPANESE APRICOT (*PRUNUSMUME*) FRUIT, FOLLOWING HEAT CONCENTRATION

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ABSTRACT

In the fruit of Japanese apricot (*Prunusmume*), many useful medicinal compounds are contained. To gain these useful medicinal components efficiently, many manufacturing technologies were developed. One of these is the heat concentration of *mume* fruit juice. Heat concentration process concentrated the useful components effectively, and can be applied as the raw material preparation technique for the processed goods. In Ehime prefecture, for example, Izumirengekai manufactured the heat concentrated juice for two locally famous *mume*'s. This technique is well established. However, the effect of heat concentration,to each chemical componentin the raw juice is not clear. Especially, to clear the change in the amygdalin content from raw juice to heat concentrated that, is one of the most attractive in the human health point of view. To clear this problem, we have analyzed the chemical component of raw and heat concentrated juices of onekind of *mume* fruit (koume) with HPLC technique, and assigned the amygdalin signals with LC/MS and LC/MS/MS techniques. With the quantitative point of view, we found that the heat concentration process used in Izumirengekaidoes not induce any significant amygdalin content change in the juice. We also compared the chemical component of concentrated stock made with whole *mume* fruit with that of concentrated juice of whole *mume* fruit, qualitatively and found that almost of the same chemical components are included in the two samples. This result strongly suggests that the boiling of whole *mume* fruit may be the alternative technique to obtain the concentrated juice containing almost the same functional components as those found in heat concentrated whole *mume* juice prepared with the traditional technique.

INTRODUCTION

The use of the fruit of *Prunusmume* has widely developed such as an herbal medicine having anti- diarrhealand antifever effects (Jee *et al*., 1999). *Prunusmume*, belongs to the family of *Rosaceae* and thought to be originated from center of China. Until now, more than 400 variations of the family of this tree are known. In addition to this medicinal effect, raw and young fruit of this tree has the poisonous effect. One of the reasons of this poisonous effect is the presence of amygdalin (Terad and Sakabe, 1988, Ohtsubo and Ikeda, 1994). Amygdalin is a sugar-containing compound and it is mainly found in immature fruits, seed and leaves of plants in*Rosaceae*, such as Japanese apricot, Japanese medlar and so on (Holzbecher *et al*., 1984, Santos *et al*., 2014).

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Digested with the enzyme such as emulsin, amygdalin is hydrolysed and hydrogen cyanide is generated. Furthermore, emulsin is the family of β-glucosidase found in animal digestive organs. For this reason, overdose of young raw *mume* (Japanese apricot) seed is thought to be toxic. On the other hand, amygdalin is known to show anti-cancer effects(Queensland Government, 2006), and also studied as the active ingredients in the traditional chinese medicine. To control this poisonous aspect of mume fruit, many manufacturing techniques have been developed. For example, in Ehime prefecture, Izumirengekai manufactured the heat concentrated juice for two locally famous *mume*'s as the raw materials for the processed goods. This technique is well established, however, the effect of heat concentration of raw juiceis not clear. To clear the effect of heat concentrationon the amygdalin content in the raw juice, with the use of HPLC, LC/MS, LC/MS/MS techniques, we have analyzed the

amygdalin content of raw and heat concentrated juices of a kind of *mume* fruit (koume). This "koume" is known as the "Nanao (re Koume" which is the regional spec（ialties, and the nutritional information analysis of this juice processed goods will induce the development of new specialties for Ehime Prefecture.We also compared the chemical component of concentrated stock made with whole *mume* fruit with that of concentrated juice, to develop a new method to prepare traditional concentrated whole mume juice much more easily.

MATERIALS AND METHODS

Reagents

For mume juice and stock sample preparation, MiliQ water was used. For HPLC, LC/MS, LC/MS/MS analyses, reagents in HPLC grades (Nakarai) were used. Amygdalin reference sample were purchased from NakaraiTesque.

Juice and stock sample preparation

"Koume", *mume* fruit, samples were collected in the farm managed by Izumirengekai. Collected samples were washed with water, and fleshes only or whole fruits including seeds, were roughly fractured with a crasher specially designed for *mume* fruits. Fractured samples were further crushed with waring blender. Crushed samples were squeezed with dish towels, and obtained juices were treated as the raw juices of *mume*fruits Raw juice samples were heat concentrated to around the 1/5 weight of original juice samples. For the heat concentrated stock sample preparation, whole *mume* fruits were boiled in the 10 times weight of water, around 2 hours. During this extraction process, volume of water was decreased around to 1/4 of original one.

Obtained raw and heat concentrated juice and heat concentrated stock samples were lyophilized, and stocked in the desiccator. For HPLC, LC/MS or LC/MS/MS analyses, to 15 mg of lyophyilized samples, 100 µl of miliQ water were added and mixed equally. This mixture was sonicated 15 min and the sample was stay overnight at 4℃. Finally, mixture was centrifuged with 20,000 x g, 15 min, and the supernatant was recovered and used as the sample for the following analyses.

HPLC, LC/MS, and LC/MS/MS analyses

LC/MS measurements were carried out in positive ion mode with a LCMS-8030 mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ion source. Precursor ions were manually selected e MS/MS analysis, and a product ion spectrum was obtained using argon as the collision gas. Other MS conditions were as follows.

CID: with -10 V,
DL/BH temperature: 250℃/400℃
Nebulizing gas flow: 2 L/min
Drying gas flow: 15 L/min

Reversed-phase HPLC (RP-HPLC) separation was performed in a C18 column (DAISOPAK SP-100-3-ODS-P, 2×150 mm; Osaka soda, Osaka, Japan). The column was eluted with 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 0.2 mL/min, using a linear gradient of 5-50% of solvent B over 20min.Column temperature was controlled at 40℃. Elution was monitored by the UV-VIS absorption in the range between 190-800 nm.

pH and sugar content

Values of pH and sugar content for each juice and stock sample were measured with a pH meter (Twin Waterpro of (B-212); HORIBA) and a saccharimeter (PAL-J; ATAGO).

RESULTS AND DISCUSSION

Assignment of the HPLC signals corresponding to amygdalin

As shown in Figure 1(a), numbers of the signals were detected, even in 257 nm monitoring. To identify the bands corresponding to amygdalin, we bought the purified amygdalin as the authentic sample, and applied to our HPLC system (blue signal in Figure 1(b)).

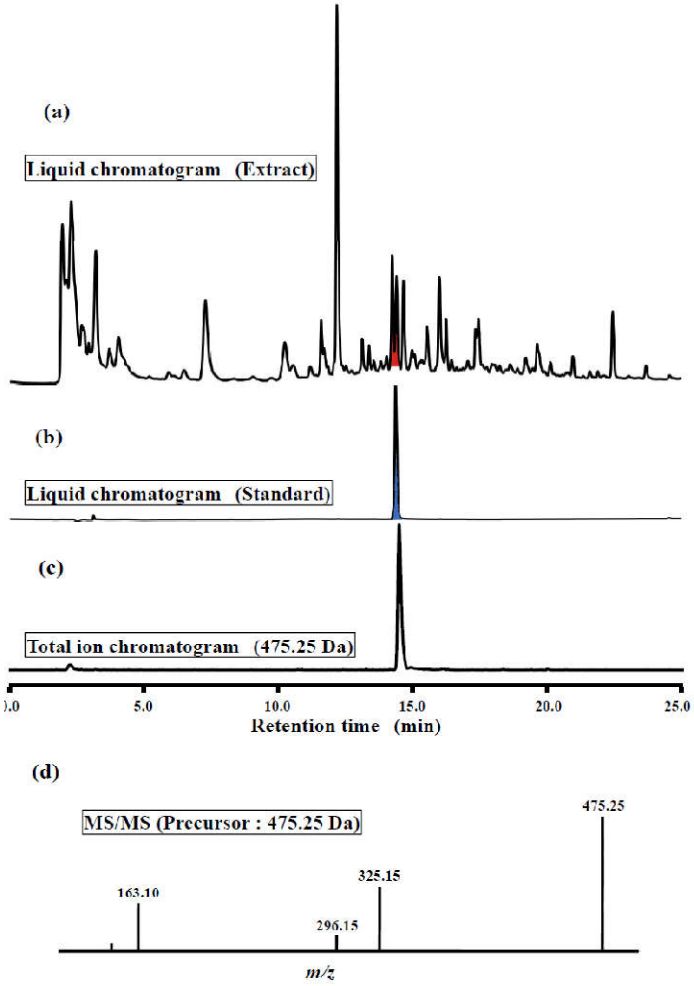


Figure 1. Fig.1 Signals originate from amygdalin in extracted *mume*samples were assigned with HPLC, LC/MS, and LC/MS/MS techniques. At first, retention times for signals found in HPLC chromatograms(a) were compared with that for reference amygdalin (b). In the next step, molecular weights of the samples contained in the speculated signals were analyzed with LC/MS technique (C) and,finally, with the use of LC/MS/MS techniques (D), we concluded that the molecules contained in the speculated signals are amygdalin

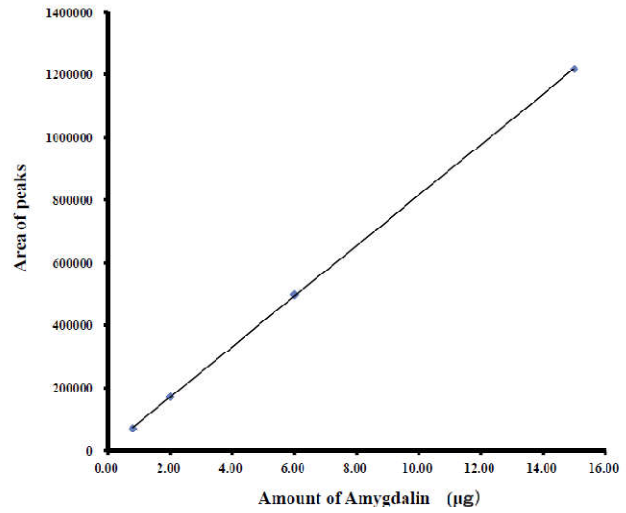
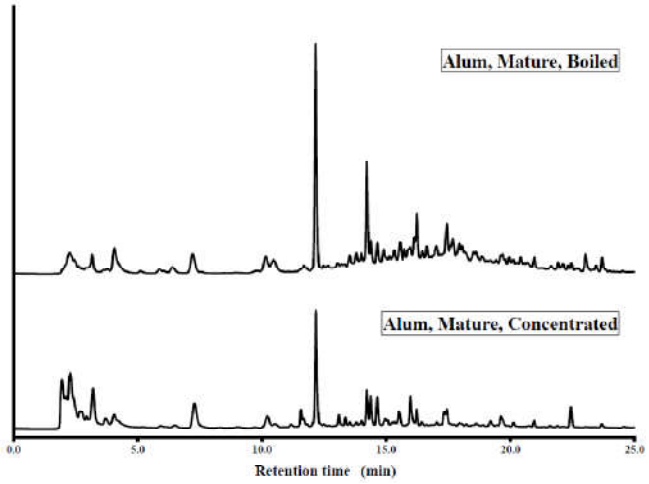
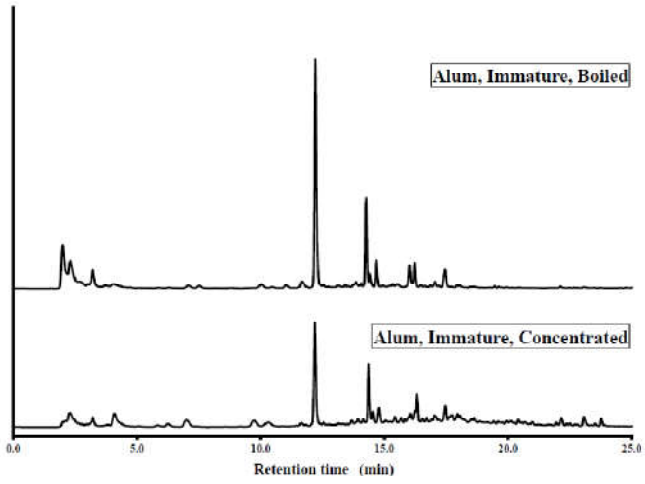


Fig.2. Standard curve between amounts of amygdalin and corresponding signal intensities in HPLC chromatogram. In this range, intensities of amygdalin signals in HPLC chromatograms and amounts of amygdalin applied to this HPLC systemwere linearly correlated



a



b

Fig.3. Comparisons of the HPLC column chromatograms between concentrated stocks and corresponding juices for a) mature, and b) immature "koume (Alum)" samples

Table 1. Amygdalin contents in 1.5mg lyophilized samples

Mature	(A) Raw juice (with seed)	2.83 µg
	Concentrated (A)	4.11 µg
	(B)Raw juice (without seed)	2.61 µg
	Concentrated (B)	3.04 µg
	Stock (concentrated)	7.69µg
Immature	(C) Raw juice (with seed)	13.07 µg
	Concentrated (C)	16.00 µg
	(D)Raw juice (without seed)	12.15 µg
	Concentrated (D)	12.14 µg
	Stock (concentrated)	10.74 µg

By comparing Figure 1(a) and 1(b), we have temporary identified the signals corresponding to amygdalin (red signals in Figure 1(a)). To identify the components in the red signals in Figure 1(a), we analyzed the molecular weights of the components in red signals with LC/MS spectrometry, and found that only the component with the molecular massin475.25 was detected (Figure 1(c)).

This result strongly suggests that the chemical compound with the molecular mass as 475.25, only presents in the red signals. This molecular mass is completely the same as that observed for authentic amygdalin sample. Molecular mass of 475.25 is larger than the molecular mass of amygdalin as 457.429 in IUPAC data. However, this difference (+17.821) may originate from the cationic form of amygdalin as $[(\text{amygdalin})+NH_4]^+$.

Furthermore, the chromatogram of LC/MS/MS analysis for the chemical compound in red signals in fig 1(a) was also the same as that for authentic amygdalin (Figure 1(d)). From these data, we concluded that the bands in red (Figure 1(a)) correspond mainly to amygdalin.

Estimation of the amygdalin content in juice and stock samples for koume

To estimate the amount of amygdalin in juice and stock samples quantitatively, we at first calibrated the relationship between the amount of amygdalin applied to HPLC system (µg scale) and the signal intensity observed in this HPLC system (integrated intensity in detection system). As shown in Figure 2, the integrated intensity of the amygdalin signal detected was linearly correlated with the amount of the amygdalin applied to this HPLC system, almost completely. In consideration of this result, we estimated the amount of amygdalin contained in the 0.3 mg of lyophilized powder from raw and concentrated juice and concentrated stock samples, as summarized in Table 1.

From this result, we found following things

- Amount of amygdalin in the juice is not changed severely, during the heat concentration process.
- Whether fruit is crashed with seed or not, amount of the amygdalin in the juice is not changed severely,

- Amount of the amygdalin is dependent on the maturation of the fruit.
- Only on the amygdalin content, concentrated stock preparation process (=only boiled whole fruit including seed,in hot water)gives us almost the same amount of heat concentrated juice preparation process for whole fruit (=crashed whole fruit including seed, and squeezed and heat concentrated).

From these finding, we concluded as follows

- In these two maturation stage, we studied, almost of all amygdalin in the seed is transferred to the flesh.
- During maturation process, amygdalin content in the fruit is decreased severely. This tendency must be induced by the presence of intrinsic emulsion in the*mume* fruit. In our studies, we did not evaluate the residual hydrogen cyanide content in the juice, however, during heat concentration process (>2hr boiling), residual hydrogen cyanide originate from the amygdalin digestion with intrinsic emulsion, must be completely decomposed, and emulsionitself also denatured during heat concentrated process. This means that the toxicity of the juice from hydrogen cyanide is completely lost, following heat treatment. Furthermore, same discussion can be applied to the process for the heat concentrated stock preparation. However, we always need to pay attention on the danger in the production of hydrogen cyanide in the digestive organs with the enzymatic effect of intrinsic □-glucosidase. This means that the overdose of green young koume fruit, even if heat treated, should be avoided

Comparison of the HPLC chromatograms between heatconcentrated juice and stock for whole fruits

In Figure 3, HPLC chromatogram for heat concentrated juice for whole fruit and that for heat concentrated stock were compared in two different maturation stages.

We found that without depending on the maturation stages of the fruits, chromatograms for two concentrated samples are almost the same. In combination with the results for the amygdalin content as discussed in the previous section, this result strongly suggest that, even only in the case for koume, the traditional protocol to prepare the heat concentrated juice for whole mume fruit (including seed) can be replaced with the simple boiling method for whole mume fruit. If this replacement is probable, safety in the processing plant will be increased. For further discussion, we will continue to study the chemical features of processedmume juice from one more different kind of *mume* fruit used in Izumirengekai,

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