Chemical Features of Phenolic Extracts Prepared on an Industrial Scale from a Processing Byproduct of the Japanese Apricot, Mume Fruit (*Prunus mume* Sieb. et Zucc.)

Takahiko MITANI1,†, Hisa MIMURA1, Asako HORINISHI2, Yoshie TANAKA3, Megumi MORI4, Nobuya INABA5, Hisako YAMANISHI4, Tomohiro AKAGI4, Takaaki OE6, Hajime KOYAMA3, Yukinori HAYASHI7, Yoshihiko OZAKI2

1 Center of Regional Revitalization, Research Center for Food and Agriculture, Wakayama University, Wakayama, Wakayama 640–8510, Japan
2 Faculty of Biology–oriented Science and Technology, Kindai University, Kinokawa, Wakayama 649–6493, Japan
3 School of Medicine, Wakayama Medical University, Wakayama, Wakayama 641–0011, Japan
4 Industrial Technology Center of Wakayama Prefecture, Wakayama, Wakayama 649–6261, Japan
5 Wakayama Agricultural Processing Research Corporation, Wakayama, Wakayama 649–6212, Japan
6 Fruit Tree Experimental Station, Wakayama Research Center of Agriculture and Fisheries, Minabe, Hidaka, Wakayama 645–0021, Japan
7 JA (Japan Agricultural Cooperatives) Kinan, Tanabe, Wakayama 646–0027, Japan

Mume fruit, Japanese apricots (*Prunus mume* Sieb. et Zucc.), have traditionally been used for pickles, juice, and liqueur in Japan. During the pickling of mume fruit, an exudate fluid from the fruit called umesu (or umezu) is produced as a byproduct. We developed laboratory-scale and factory-scale methods using synthetic absorbents HP-20 column chromatography to prepare phenolic fractions from umesu (umesu phenolics or umezu phenolics, hereafter referred to as UPs). In this study, we obtained six batches of UPs using a factory-scale method, and their chemical features were examined. The phenolic contents were 13.1±0.8% and 19.2±2.1%, respectively, with gallic acid and p-coumaric acid as standards. The total sugar content was 57.7±4.7%. Very close similarity was observed in the high-performance liquid chromatograms and compositions of the phenolics in the six batches of UPs. The four major phenolic compounds found in the alkaline hydrolysate of UPs were caffeic acid, cis-p-coumaric acid, trans-p-coumaric acid, and ferulic acid. Some batches showed high amounts of cis-p-coumaric acid / total p-coumaric acid. Since this isomerization did not occur during the UP preparation process, it seems likely that isomerization of trans-p-coumaric acid into cis-p-coumaric acid occurred due to sunlight irradiation during umesu storage.

**Keywords:** *Prunus mume*; umeboshi; byproduct; phenolics

1. Introduction

Mume fruit, the Japanese apricot (*Prunus mume* Sieb. et Zucc.), belongs to the *Rosaceae* family and is one of the most popular fruits in Japan, with an annual production of ~100,000 tons. In Japan, mume fruit is processed into umeboshi (pickled mume fruit), umeshu (liqueur), juices, etc. Processed mume fruit products have traditionally also been used as a folk remedy in Japan and eastern Asian countries [1]. Several investigations have been conducted into the constituents of mume fruit that might provide health benefits. Chlorogenic acids, flavonol oligoglycosides, prunoses I, II, and III, and mumeose K-O, all found in the fruit or flowers of mume trees, were reported to show several physiological activities [2-8].

We have already found that the flesh of fully mature mume fruit contains up to 1% of phenolic compounds on a dry-weight basis and demonstrated that the phenolics in the methanol-extractable fraction were hydroxycinnamic acid (mainly p-coumaric acid, caffeic acid, and ferulic acid) derivatives. Among these, 3 compounds have been identified as chlorogenic acid (5–O–caffeoylquinic acid), 3–O–caffeoylquinic acid, and Prunose II [9].

During the umeboshi production process, the harvested fruit is first salted and pressed for a few weeks. In the process of pickling, the salted fruit gradually exudes its juice, called umesu or umezu (often translated as...
‘mume vinegar,’ although it is not true vinegar), one of the major byproducts of umeboshi making. Since umesu contains high concentrations of NaCl (20%) and citric acid (5%), it is difficult to find a proper use or application for this byproduct. However, umesu is expected to contain aqueous phenolic compounds because it is exuded from mume fruit.

We have already prepared phenolic fractions in the laboratory from umesu (umesu phenolics, UP) derived from the 2006–2008 harvest seasons by using synthetic adsorbents HP-20 (Mitsubishi Chemical Corporation) and found that the three batches of UP demonstrated very close similarity in their high-performance liquid chromatography (HPLC) results and compositions of phenolic compounds [10]. In laboratory studies, several approaches were used in order to scale-up the production of UP. These included increasing the following: (1) amount of phenolic compounds that adhere to HP-20 column, (2) efficacy of water washing for elimination of sodium chloride and citric acid in the column, (3) recovered amount of phenolic compounds in 60% aqueous ethanol eluate and (4) regeneration of HP-20 resin after chromatography. These experiments were useful for the establishment and validation of a scaled-up production. However, the majority of umesu is obtained at umeboshi manufacturing farms in Wakayama Prefecture. Therefore, it was important to characterize the bulk UP, considering that there could be large variations in the quality of umesu from different orchards.

Recently, we have been able to obtain UPs in bulk from various batches of umesu. In this paper, we report analytical data for the mass-produced UPs and discuss the quality of umesu from different orchards.

### 2. Materials and Methods

#### 2.1 Chemicals

Caffeic acid, trans-\(p\)-coumaric acid, and ferulic acid were purchased from Sigma–Aldrich (St Louis, MO, USA) or Wako Pure Chemical Industries Ltd. (Osaka, Japan). Diaion HP-20 resin was obtained from Mitsubishi Chemical Co. Ltd. (Tokyo, Japan). Folin–Ciocalteu reagent was purchased from Nacalai Tesque (Kyoto, Japan). Diaion HP-20 resin was obtained from Mitsubishi Chemical Co. Ltd. (Tokyo, Japan). Folin–Ciocalteu reagent was purchased from Nacalai Tesque (Kyoto, Japan). Diaion HP-20 resin was obtained from Mitsubishi Chemical Co. Ltd. (Tokyo, Japan). Folin–Ciocalteu reagent was purchased from Nacalai Tesque (Kyoto, Japan).

#### 2.2 Samples

Six UP batches (#1, #2, #3, #4, #5, and #6), produced in bulk, were obtained. UP#1 was a product of TOKIWA Phytochemical Co. Ltd., Chiba, Japan, and UP#2 and UP#3 were donated by SUNACTIS Co. Ltd., Osaka, Japan. UP#4, UP#5, and UP#6 were gifts from Kinan Agricultural Cooperatives (Tanabe, Wakayama).

### 2.3 Determination of total phenolics and sugar

The total phenolic content was determined by the Folin–Ciocalteu method with gallic acid as a standard [11]. \(p\)-Coumaric acid was also used as a standard. The total sugar content was assayed by the phenol–sulfuric acid method with glucose as a standard [12].

The moisture was expressed as weight loss upon drying at 90°C for 24 h under atmospheric conditions.

### 2.4 HPLC analyses

A Shimazu LC-2010 instrument was used for HPLC. All samples were injected into a HydroSphere C18 column (ø4.6 mm × 250 mm; YMC, Kyoto, Japan). The column was eluted with mixed solvents A (0.1% trifluoroacetic acid in water): B (methanol) at a flow rate of 1.0 mL/min. The eluent was A: B = 80:20 for 10 min, then a linear gradient system was applied that reached A: B = 25:75 after 80 min. All analyses were carried out at 30°C and monitored by UV absorption at 280 nm.

### 2.5 Method for UP preparation in bulk

In the preparation of UPs, Diaion HP-20 resin, which is often used to prepare phenolic extracts, was used [13,14]. An example of the column method on the industrial scale was as follows: 3000 L of umesu were applied to a Diaion HP-20 column (ø30 cm × 2 m) at a flow rate of 300 L/h, and the column was washed with 500 L water to remove sodium chloride and citric acid for 1 h. The phenolic compounds were eluted with 500 L of 60% aqueous ethanol containing 0.05% acetic acid over 1 h. The eluate was then concentrated under vacuum at 50°C, freeze-dried, and stored at –30°C until analysis.

### 2.6 Alkaline hydrolysis

Ester linkages were hydrolyzed by alkali as described by Krygier et al. [15]. Samples of 100 mg of UP or 250 mg of freeze-dried fruit flesh were suspended in 4 mL of 1 N NaOH that were deoxidized in advance by nitrogen gas bubbling. After 4 h of incubation at 37°C, the suspension was acidified by adding phosphoric acid and was extracted three times with ethyl acetate. The combined ethyl acetate layers were evaporated to dryness, and the residue obtained was dissolved in a minimal volume of
HPLC mobile phase. The contents of caffeic acid, p-coumaric acid, and ferulic acid in the UP hydrolysate were quantified by comparison with the HPLC peak areas of authentic compounds.

### 2.7 Preparative HPLC

An AKTA Explorer 10S System (GE Healthcare Bioscience) was used for preparative HPLC. A portion of UPs was loaded into a Hydrosphere C18 column (ø10 mm×250 mm, 5 μm; YMC, Kyoto, Japan). Separation was performed by elution with a series of linear gradients of C (0.5% formic acid in water) and D (methanol) in a cold room at 4℃ with a flow rate of 1.5 mL/min as follows: 15% D in C; 15–35% D in C, 0–20 min; 35–100% D in C, 220–260 min. Aliquots of elute corresponding to each peak were collected, evaporated to dryness, and dissolved in methanol.

### 2.8 Determination of NMR spectra

The isolated sample was dissolved in methanol-\(^{d_4}\) or deuterated dimethylsulfoxide. The \(^1\)H NMR and \(^{13}\)C NMR spectra were recorded on a Bruker Avance 400 MHz (100 MHz \(^{13}\)C) spectrometer. Chemical shifts are reported as δ values (in ppm) relative to internal tetramethylsilane and coupling constants (J) are given in Hertz.

### 2.9 Spectral data of isolated compounds from UP

**Compound A, caffeic acid:** \(^1\)H NMR (400 MHz, methanol-\(^{d_4}\)): δ=6.21 (1H, d, J=16 Hz), 6.76 (1H, d, J=8 Hz), 6.92 (1H, dd, J=2, 4 Hz), 7.02 (1H, d, J=4 Hz), 7.52 (1H, d, J=16 Hz).

**Compound B, cis-p-coumaric acid:** \(^1\)H NMR (400 MHz, methanol-\(^{d_4}\)): δ=5.77 (1H, d, J=16 Hz), 6.73 (1H, d, J=8 Hz), 6.77 (1H, d, J=8 Hz), 7.59 (1H, d, J=8 Hz).

**Compound C, trans-p-coumaric acid** [9]: \(^1\)H NMR (400 MHz, methanol-\(^{d_4}\)): δ=6.28 (1H, d, J=16 Hz), 6.80 (2H, d, J=8 Hz), 7.44 (1H, d, J=8 Hz), 7.59 (1H, d, J=16 Hz).

**Compound D, ferulic acid:** \(^1\)H NMR (400 MHz, methanol-\(^{d_4}\)): δ=3.89 (3H, s), 6.31 (1H, d, J=16 Hz), 6.81 (1H, d, J=8 Hz), 7.06 (1H, dd, J=4, 8 Hz), 7.17 (1H, d, J=4 Hz), 7.59 (1H, d, J=16 Hz).

### 3. Results and Discussion

#### 3.1 Chemical analysis of UPs on an industrial scale

The moisture, phenolics, and total sugar contents of six batches of UPs produced on an industrial scale are shown in Table 1. There was not much of difference in the contents of moisture, phenolics, and total sugars among the six batches. It was assumed that the 20% sodium chloride and 5% citric acid contained in umesu provided a favorable environment to stabilize the phenolic compounds. Oe et al. reported that phenolic contents in mume fruits are relatively stable annually under different weather conditions [16].

Gallic acid, which has three hydroxide groups, is usually commonly used as a standard in the Folin–Ciocalteu method. The phenolics of mume fruit consist of p-coumaric acid, caffeic acid, and ferulic acid, which have one or two hydroxide hydroxyl groups in each molecule [9]. Therefore, if gallic acid is used as the standard, the total phenolic content of the UPs should be underestimated. Hirawan et al. reported the Folin–Ciocalteau method for assessment of phenolic acids in wheat by using ferulic acid as the standard [17]. As shown in Table 1, shows that the total phenolic content of UPs with p-coumaric acid as the standard was about ~48% higher than that with gallic acid as the standard.

HPLC chromatograms of the UPs are shown in Fig. 1A. Each HPLC chromatogram was broadly similar to the others, but there were some peaks in a different portion of the chromatogram. These variations reflect small structural modifications of the phenolics due to the orchard location and picking season of the mume fruit or the storage conditions for the umesu.

### Table 1 The content of moisture, phenolics, and sugar in UPs prepared in bulk.

<table>
<thead>
<tr>
<th></th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
<th>#4</th>
<th>#5</th>
<th>#6</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>3.25</td>
<td>2.49</td>
<td>3.56</td>
<td>4.52</td>
<td>3.46</td>
<td>2.22</td>
<td>3.25±0.12</td>
</tr>
<tr>
<td>Phenolics GA (%)</td>
<td>14.4</td>
<td>12.0</td>
<td>12.8</td>
<td>12.8</td>
<td>13.3</td>
<td>14.5</td>
<td>13.1±0.8</td>
</tr>
<tr>
<td>Phenolics PC (%)</td>
<td>17.4</td>
<td>20.8</td>
<td>16.7</td>
<td>19.4</td>
<td>22.4</td>
<td>18.7</td>
<td>19.2±2.1</td>
</tr>
<tr>
<td>Total sugar (%)</td>
<td>52.1</td>
<td>56.6</td>
<td>53.7</td>
<td>63.7</td>
<td>62.6</td>
<td>56.0</td>
<td>57.7±4.7</td>
</tr>
</tbody>
</table>

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3.2 Alkaline hydrolysis of UPs and characterization of isolated compounds from alkaline hydrolysate of UPs

Many peaks were observed in the chromatograms of the UP as shown in Fig. 1A. We have already reported that alkaline hydrolysis simplified the complex phenolic profile of mume fruit [9]. As shown in Fig. 1B, the chromatogram of the hydrolysate for each batch of UPs consisted commonly of four major peaks and several minor peaks.

The four major peaks (Peaks 1, 2, 3, and 4) were purified using preparative HPLC, and the chemical structure corresponding to each peak was confirmed by comparing the HPLC retention time and $^1$H NMR spectrum with...
those of standard compounds. When a standard compound was not commercially available, the 1H NMR spectrum was compared with those in the literature. Peaks 1, 2, 3, and 4 were assigned to caffeic acid, cis-p-coumaric acid, trans-p-coumaric acid, and ferulic acid, respectively [9].

These results showed that the UPs consisted of hydroxycinnamic acid derivatives and that these four compounds were identical to those in the phenolics of mume fruit. As the ethanol-extractable fraction of the kernel and albumen did not contain caffeic acid, cis-p-coumaric acid, trans-p-coumaric acid, and ferulic acid (data not shown), it was concluded that the UPs were derived from the mume fruit flesh.

### 3.3 Occurrence of cis-p-coumaric acid in Ups

We have already reported the presence of cis-p-coumaric acid in mume flesh phenolics [9]. Table 2 indicates the ratio of cis-p-coumaric acid to total p-coumaric acids (cis-p-coumaric acid + trans-p-coumaric acid) in the six UP batches and shows that there was a large variation in the ratio. We have tried to resolve the cause of this variation. Among the six UP batches, it was clarified that the umesu for UP#2, UP#3, and UP#4 had been kept in translucent containers out of doors for several months. On the other hand, the umesu for UP#1, UP#5, and UP#6 had been stored in containers inside buildings. Since it is known that ultraviolet light induces isomerization of trans-p-coumaric acid to cis-p-coumaric acid, we attempted to determine whether such isomerization was induced in umesu kept in translucent containers out of doors. An aliquot of umesu from UP#5 in a clear plastic container was left under sunlight irradiation for two months. The phenolic fraction was prepared from the umesu by using HP-20 column chromatography and was hydrolyzed by alkali. As shown in Fig. 2, the peak assigned to cis-p-coumaric acid had increased in height after sunlight irradiation. It is thought that the high percentage of cis-p-coumaric acid in UP#2, UP#3, and UP#4 resulted from their storage outdoors. The ratios of cis-p-coumaric acid to total p-coumaric acids in the umesu for UP#5 and UP#6 were 22.8% and 25.5%, respectively. Therefore, we believe that the isomerization occurred due to the quality of umesu, not by the UP-preparation process. Since we have obtained preliminary data in which there seems to be a difference in the bioavailability of p-coumaric acid between the cis and trans forms, it is important to store the umesu out of sunlight. Mäkilä et al., reported that trans-p-coumaric acid is more easily converted into the corresponding cis isomer under UV or sunlight than caffeic and ferulic acid [18]. Therefore, cis-p-coumaric acid was detected in the UPs.

These chemical analyses will contribute to the produc-

<table>
<thead>
<tr>
<th>UP#</th>
<th>cis-p-coumaric acid (%)</th>
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<tr>
<td>1</td>
<td>18.7</td>
</tr>
<tr>
<td>2</td>
<td>39.6</td>
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<td>3</td>
<td>34.6</td>
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<tr>
<td>4</td>
<td>37.4</td>
</tr>
<tr>
<td>5</td>
<td>22.8</td>
</tr>
<tr>
<td>6</td>
<td>25.5</td>
</tr>
</tbody>
</table>

Fig. 2. Isomerization of p-coumaric acid in umesu after sun irradiation for two months. An aliquot of umesu was kept in translucent containers outdoors for two months. The phenolic fraction was prepared from the umesu by using HP-20 column chromatography and was hydrolyzed by alkali. The hydrolysates were analysed by HPLC. (A) 0 months. (B) 2 months.
tion of Ups, which that may be useful as a food ingredients in health-promoting foods.

Acknowledgements

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ウメ（Prunus mume Sieb.et Zucc.）果実の加工副産物から工業的に調製したフェノール性化合物の化学的性質

三谷隆彦1,†, 味村妃紗1, 堀西朝子2, 多中良栄3, 森めぐみ4, 稲葉伸也5, 山西妃早子4, 赤木知裕4, 大江孝明6, 小山一3, 林行則7, 尾崎嘉彦2

1和歌山大学 地域活性化総合センター 食農総合研究所, 2近畿大学生物理工学部, 3和歌山県立医科大学, 4和歌山県工業技術センター, 5一般社団法人和歌山県農産物加工研究所, 6和歌山県果樹試験場 うめ研究所, 7紀南農業協同組合

ウメ（Prunus mume Sieb. et Zucc.）果実は梅干し,ジュース,梅酒などに加工されている. 和歌山県はウメ果実の生産量は約6万トンで,全国の生産量の6割に達し,その70%が県内で梅干し製造に用いられる. 梅干し製造過程では推定1.6万トンの梅酢が副産物として生じるが,梅酢は20%の食塩と5%のクエン酸を含有していることから,その利用,もしくは廃棄法が大きな課題となっている. われわれは梅酢には果実由来のフェノール化合物が存在し,その調製法を実験室レベルで明らかにしてきた. その後, 梅酢由来のフェノール化合物(UPと省略)を工業的に製造する開発に繋がった. その方法の1つを示すと, 化学吸着樹脂Diaion HP-20を充填したステンレスカラム（直径30cm×長さ2m）に梅酢3トンを流速300L/hで通過させ, フェノール性化合物を吸着させる. その後脱イオン水500Lを通過させてカラム内に残存している食塩およびクエン酸を除去する. その後500Lの0.05%の酢酸を含む60%のエタノールをカラムに流し, フェノール化合物を回収する. この液を凍結乾燥してUP原末を得る. UPの工業的製造が開始されたので, 工業的に製造した6ロットの化学的特性を調べた. フェノール性化合物の平均含量をフォーリン・チオカルト法で調べた. この場合, 通常没食子酸を検量用の標準物質として用いるが, 没食子酸はフェノール環に水酸基が3個ついている. ところでウメのフェノール性物質はヒドロキシ桂皮酸の誘導体で, フェノール環に水酸基は1個もしくは2個であることがすでに明らかになっている. 没食子酸を標準物質にすると, フェノール性物質の含量が低く見積もられることになる. そのため, フェノール環に水酸基が1個のp-クマル酸も, 別途標準物質として用いた. その結果, UPのフェノール性化合物の平均含量は, 没食子酸換算で13.1±0.79%, p-クマル酸換算で19.2±2.11%であることが判明した. 全糖量はフェノール硫酸法で求め, 其の平均含量は57.7±4.7%であった. これらの6ロットのHPLCプロフィアルおよびフェノール性化合物の組成はピークの高さは多少の違いはあるものの, 全体として類似していた. UPのアルカリ加水分解によりカフェ酸, trans-p-クマル酸, cis-p-クマル酸, およびフェルラ酸が見いだされた. これらは実験室レベルで製造したUPと, 同じ分子種であった. これまで, 実験室レベルで製造したUPは, 梅酢製造の年が違っても, またウメ果実の収穫場所が異なっても, 品質に大きな差異は見いだされなかったことから, 工業的なレベルでもこの事事が確認できた. 恐らく梅酢中では塩分濃度が高く, 酸性条件であるためフェノール性化合物は長期間安定に保たれると考えられた. 以上, これらの検査項目はUPの品質規格として利用される予定である. しかし工業的に製造したUPの浅つかのロットでは, p-クマル酸総量に占めるcis-p-クマル酸の割合が高いものが見いだされた. メンツ干し製造後に生じた梅酢は, 個々の梅酢製造業者や農家で保管されているが, 其の保管方法はバラバラで, 屋外や屋内で, 半透明もしくは光を通さない容器で保管されている. cis-p-クマル酸量はtrans-p-クマル酸が紫外線の作用を受けることで異性化されるので, ウメ果実でも一定量が生成していると考えられるが, UPの中で極端にその割合が高いロットは, 原料梅酢が太陽光に当たる場所で半透明の容器に保管されていたことが追跡調査で明らかになった. この事実を再現するため, 実験室レベルで梅酢を太陽光に2か月間照射した場合, p-クマル酸総量に占めるcis-p-クマル酸の割合が著しく増加したことから, 今後工業的にUPを製造する場合, 原料梅酢の保管方法を十分考慮する必要がある.