A REFERRED PAPER

ANALYSIS OF THE PHENOLIC COMPOUNDS
IN LONGAN (DIMOCARPUS LONGAN LOUR.) PEEL

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Abstract. Longan fruit are susceptible to chilling injury, where the
injured peel exhibits discoloration due to water-soaking and enzymatic
browning. This peel discoloration is dependent to a large degree on the
composition of the phenolic compounds. Yet, the main classes of phenols in longan peel re-
main largely uncharacterized. In order to further characterize these classes of compounds, and to begin investigating the
differences in these compounds resulting from chilling injury, two varieties (‘Daw’ and ‘Biew Kiew’) were subjected to
cold storage. Peels of the stored fruit were freeze-dried and extracted
with 80% aqueous methanol. HPLC, coupled with photo-
diode array (PDA) and mass spectral (MS) analysis, provided
evidence of a large number of UV-absorbing compounds in
longan peel, the majority of which are phenolic compounds.
Subsequent fractionation of these compounds by size-exclusion
chromatography effectively separated several main
classes of phenolic compounds, including ellagic acid conju-
gates, flavone glycosides, and a set of other phenols with un-
known structures, but which were grouped together because of
their similar MS, UV and IR spectra. HPLC analysis of acid
hydrolyzed extracts of longan peel showed that the majority of
the flavones were glycosides of quercetin and kaempferol.
Similar analyses also showed evidence of ellagic acid glyco-
sides. The spectral properties of these compounds are report-
ed, and several of the main classes of phenols in longan peel are
described.

Longan (Dimocarpus longan Lour.) is a tropical, non-cli-
macteric fruit that has a short shelf-life, and must be harvested
when their skins become yellow-brown and their flesh
reaches optimal eating quality (Pan and Zhang, 1999; Wara-
Aswapati et al., 1994). Furthermore, longan fruit are suscepti-
able to chilling injury, where the peel exhibits discoloration
due to water-soaking and browning (Wang, 1990). Rates of
peel browning have been shown to be dependent upon the
concentrations of the phenolic compounds, the enzymatic ac-
tivity of polyphenol oxidase, and oxygen levels (Kader, 2002;
Lin et al., 2005; Nicolas et al., 1994; Su et al., 2005). The pheno-
lols in longan peel have been previously reported to include
gallic acid, ellagic acid, acetonylgeraniin A and B, and corilla-
gin (an ellagitannin) (Hsu and Chyn, 1991; Rangkadilok et
al., 2005). Yet in this current study, HPLC-PDA-MS analyses of
longan peel extracts show far greater numbers of compounds
than those previously described. The objective of this study
was to provide an initial characterization of the main classes
of phenols in the peel of chilling-injured Daw and Biew Kiew
longan fruit. This characterization will contribute to the un-
derstanding of the biochemical transformations in the peel
resulting from chilling injury during postharvest storage.

Materials and Methods

Fruit source. Longan fruits cv. ‘Daw’ and ‘Biew Kiew’ were
harvested from a commercial orchard in northern Thailand.
The maturity index of fruit was 18-19% total soluble solids.
Uniform fruit were selected and the pedicles were cut into 0.5
cm in length. Two kilograms of fruit were packed in card-
board boxes and stored at 5°C or 10°C in an incubator (Low
temperature incubator, MIR-553, SANYO Electric Co., Ltd.,
Japan), with 90-95% relative humidity (RH ) using a data log-
ger (Temperature and Humidity Recorder, HiTemp 102 RH,
Barnstead/ ERTCO Thermometers Co., Inc., USA) for re-
cording temperature and RH. Three replicates of 20 fruits
were used for each control and chilling-injury treatment. The
longan fruit pericarpers were submerged in liquid nitrogen and
stored at -80°C (Deep Freezer -86°C, MDF-U71V, SANYO, Ja-
pan) until extraction for chemical analysis.

Sample extraction. Peel (10 g) of chilling-injured and of
non-chilling injured longan fruits (cv. ‘Daw’ and ‘Biew Kiew’)
were freeze-dried and refluixed in 200 mL 80% aqueous meth-
nol for 24 h. The extracts were concentrated to 15 mL with
a rotary evaporator at 40°C under reduced pressure.

Size-exclusion P2 chromatography. Peel extracts from 15 fruit
were combined and dried by SpeedVac concentration (Sa-
vant, Farmingdale, N.Y.). The dried, combined extract was
dissolved in 20% aqueous ethanol, filtered through a 0.45 µm
syringe filter, and applied to a Biogel P2 (BioRad, Hercules,
Calif.) size-exclusion column (30 × 2.5 cm i.d.). The phenolic
compounds were eluted with 20% aqueous ethanol at 0.5
mL·min⁻¹. Elution of the phenols was monitored at 280 nm,
and phenolic content was monitored by HPLC-PDA-MS in ev-
ery 3rd tube.

Acid hydrolysis. Acid hydrolysis was performed according to
Mabry et al. (1970) with slight modifications. P2 column frac-
tions (5 mL) were added to 5 mL of 2N HCl, heated at 100°C
for 1 h, cooled, and neutralized with 2.5 N KOH. The hydro-
dylized samples were concentrated by a SpeedVac concentra-
tor for 1 h, and passed through a C18 Sep-Pak (Waters Corp., Mil-
ford, Mass.) size-exclusion column (30 × 2.5 cm i.d.). The phenolic
compounds were eluted with methanol and analyzed by HPLC-PDA-MS.

High pressure liquid chromatography-mass spectrometry (HPLC-
MS). An Alliance 2695 (Waters, Milford, Mass.) liquid chro-
matography system was used with a Waters 996 PDA detector.
Spectra were scanned between 600 and 200 nm. Chromatograms were monitored at both 330 nm and 280 nm. A Waters ZQ single quadrupole mass spectrometer with an electrospray interface was used in conjunction with the above HPLC system. Interface parameters were: source temperature 100°C, desolvation temperature 225°C, capillary voltage 3.33 kV, cone voltage 20 V, extractor voltage 2 V, RF lens voltage 0.1 V, desolvation gas flow 465 L·h⁻¹, and cone gas flow 70 L·h⁻¹.

Chromatographic separations were obtained with a C16 Discovery RP Amide column (25 cm × 4.6 mm, 5 µm) (Supelco, St. Louis, Mo.). Initial solvent conditions were water/ acetonitrile/2% formic acid (85/10/5 v/v/v). Linear gradients were subsequently run to (81/14/5), (77/18/5), (70/25/5), (40/55/5), and (0/95/5) at 15, 20, 30, 55, 67 min, respectively, at a flow rate of 0.75 mL·min⁻¹. Chromatograms were monitored at 330 nm. Data acquisition was done with MassLynx v. 3.5 software.

Isolation of phenolic unknowns. P2 size-exclusion column fractions that contained a set of 4 unknown phenols that exhibited similar UV and MS properties were combined and dried by rotary evaporation under vacuum. The dried fraction was dissolved in methanol, and loaded onto tapered silica gel GF preparative TLC plates with fluorescence indicator (Alltech, Newark, Del.). Plates were developed twice in butanol/ acetic acid/ water (65/15/20 v/v/v). This solvent system provided a broad separation of the compounds in these P2 column fractions. Lanes detected with 365 nm UV irradiation were scraped, extracted with methanol, and analyzed by HPLC-PDA-MS. The targeted unknown phenols were identified in a single lane, and were further isolated by preparative HPLC. Chromatographic separations were obtained with a Waters Delta 600 preparative HPLC system, using a C18 Atlantis column (10 cm × 19 mm, 5 µm) (Waters, Milford, Mass.). Initial solvent conditions were water/ acetonitrile/2% formic acid (85/10/5 v/v/v). Linear gradients were subsequently run to (81/14/5), (77/18/5), (70/25/5), (40/55/5), and (0/95/5) at 15, 20, 30, 55, 67 min, respectively, at a flow rate of 5.0 mL·min⁻¹. Chromatograms were monitored with a Waters 996 PDA detector at 330 nm. Separate peaks (4) were collected, dried, and analyzed for purity on analytical silica gel TLC using butanol/ acetic acid/ water (65/15/20 v/v/v).

Results

The phenolic compounds in the peel of two varieties of longan (‘Daw’ and ‘Biew Kiew’) were analyzed by HPLC-PDA-MS. These analyses were conducted on the peel of untreated fruit and on chilling-injured fruit. These initial analyses showed a large number of UV-absorbing compounds in the peel extracts, but did not show any clearly distinguishable differences between the peel of the control and chilling-injured fruit of either variety of longan (data not shown). Part of this failure to detect any clear differences was due to the large number of peak overlaps in the HPLC chromatograms, and hence, accurate UV (PDA) and MS analyses of many of the compounds in the longan peel were not possible.

To achieve HPLC peak separations sufficient for accurate UV (PDA) and MS analysis, the phenolic compounds in these extracts were fractionated by Biogel P2 size-exclusion chromatography. This resulted in the resolution of several classes of phenolic compounds, including ellagic acid conjugates, flavonoid glycosides, and numerous other unknown compounds. Figure 1 shows the HPLC chromatograms of three sequential P2 column fractions in which these classes of compounds were detected. The initial-eluting P2 column fractions (data not shown) contained broad, poorly resolved sets of compounds, similar to those previously described in early-eluting size-exclusion LH20 chromatographic fractions of longan seed extracts (Soong and Barlow, 2005). The subsequent-eluting P2 column fractions contained ellagic acid conjugates and a specific group of unknown compounds (Fig. 1A, B). Later eluting fractions contained flavone glycosides (Fig. 1C). Other compounds in these main classes of phenols were similarly detected in other fractions (data not shown).

Figure 2 shows the UV and MS of the major ellagic acid conjugates detected in the freeze-dried longan peel. The compounds were initially identified by their UV spectra, which matched that of an ellagic acid standard. The compounds were further detected by the presence of the mass ion at 303 m/z, which matched the protonated molecular weight (M+H)⁺ of ellagic acid. The 303 m/z ion was particularly evident at higher cone voltages (fragmentation energies) i.e. 60 V, whereas at the lower cone voltage, i.e. 20 V, higher mass ions were detected that were consistent with the neutral losses of hexoses (146 amu for possible rhamnose), and a pentose (132 amu). The MS data indicate that the main ellagic acid conjugates in longan peel are glycosides.

![Fig. 1. HPLC chromatograms at 330 nm of three P2 column fractions. Ellagic acid conjugates in chromatograms A and B are labeled by EA, and a set of related phenolic unknown compounds is labeled with (*) in chromatogram A. The quercetin glycosides and kaempferol glycosides are labeled by Q and K in chromatogram C, respectively.](Image)

![Fig. 2. UV and mass spectra of the major ellagic acid conjugates detected in the freeze-dried longan peel.](Image)
HPLC-PDA-MS analysis of the later-eluting P2 column fractions showed the presence of a number of flavonoid glycosides. Two classes were identified, including quercetin glycosides and kaempferol glycosides. Acid hydrolysis of longan peel extracts produced two flavone aglycones, which when analyzed by HPLC-PDA-MS exactly matched the elution times, UV, and MS of authentic samples of quercetin and kaempferol (data not shown).

The quercetin glycosides were detected by their UV spectra, which were characteristic of numerous previously reported quercetin glycosides (Mabry et al., 1970), and by the presence of a mass ion of 303 m/z, which matched the protonated molecular weight (M+H)$^+$ of quercetin (Fig. 3). Three of the compounds (A-C) exhibited higher mass ions suggesting the neutral losses of hexoses (162 amu of possible glucose [(465 amu - 303 amu), in Fig. 3B, C] and 146 amu for rhamnose (Table 1).

<table>
<thead>
<tr>
<th>Elution time (min)</th>
<th>MS</th>
<th>Possible structure</th>
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<tbody>
<tr>
<td>21.1</td>
<td>617(+Na)/595(M+H)$^+$ + 287(-162)</td>
<td>K + glu + rha</td>
</tr>
<tr>
<td>23.1</td>
<td>617(+Na)/595(M+H)$^+$ + 287(-162)</td>
<td>K + glu + rha</td>
</tr>
<tr>
<td>24.4</td>
<td>471(+Na)/449(M+H)$^+$ + 287(-162)</td>
<td>K + glu</td>
</tr>
<tr>
<td>26.1</td>
<td>441(+Na)/419(M+H)$^+$ + 287(-132)</td>
<td>K + pentose</td>
</tr>
<tr>
<td>28.7</td>
<td>455(+Na)/433(M+H)$^+$ + 287(-146)</td>
<td>K + rha</td>
</tr>
</tbody>
</table>

Table 1. Mass spectra of kaempferol glycosides in longan peel. Abbreviations are: K, kaempferol; glu, glucose; rha, rhamnose. A pentose is indicated by the neutral loss of 132 amu (150 amu - 18 (H$_2$O)). The +Na adduct (+Na) is observed as a mass ion 22 amu above the protonated mass ion (M+H)$^+$. Neutral losses indicated by parentheses.

Fig. 3. UV and mass spectra of quercetin glycosides in freeze-dried longan peel.

Fig. 4. UV and mass spectra of 2 kaempferol glycosides in freeze-dried longan peel.

Fig. 5. Analytical silica gel TLC of 3 related unknown compounds. The 3 main unknown compounds, A-C, were applied to 200 micron thickness Analytech analytical TLC plates with fluorescence indicator. Visualization was made with UV light (365 nm), and with the application of a 5% H$_2$SO$_4$ in ethanol spray followed by heat. No spots in lanes A-C other than the unknown compounds were detected by either UV irradiation or by charring with H$_2$SO$_4$ and heat.
nose [(449 amu - 303 amu) in Fig. 3A]). The two remaining compounds (D and E) each exhibited two higher mass ions, suggestive of the neutral losses of both a glucose and a rhamnose moiety. An additional compound exhibited fragment ions at indicative of a quercetin trisaccharide (Q + glucose + 2 rhamnose) (data not shown).

The UV and MS of two of the main kaempferol glycosides are shown in Fig. 4. Both compounds exhibited UV spectra characteristic of previously reported kaempferol glycosides (Mabry et al., 1970), and mass ion fragments at 287 m/z, matching the protonated molecular weight (M+H)$^+$ of kaempferol. The MS of compound A shows the neutral loss (419 amu - 287 amu) of 132, possibly a pentose, while compound B shows the neutral loss (449 amu - 287 amu) of 162, matching a hexose, possibly glucose. As shown in Table 1, three additional kaempferol glycosides were detected, including a rhamnoside (shown by a neutral loss of a single 146 amu fragment), and 2 disaccharides (both exhibiting neutral losses of 162, and 146, possibly glucose and rhamnose, respectively).

The longan peel extracts further contained a large number of compounds that belied easy structural analysis by HPLC-PDA-MS. A particularly interesting set of 4 compounds eluted between 26-28 min (Fig. 1A), and exhibited nearly identical UV and mass spectra. Further analysis was done on thin layer chromatography (TLC) and HPLC purified samples of these compounds. The three major compounds in this set of unknowns exhibited nearly identical migration on TLC and were isolated to a single spot on analytical TLC (Fig. 5). Although the recovered amounts were not sufficient for NMR analysis, there were sufficient amounts for further MS analysis and FTIR spectroscopy. Figure 6 shows the UV and MS of the 3 isolated compounds.

The UV spectra were nearly identical, and were similar to UV spectra of hydroxycinnamates (Wolf 1968). In the same manner, the MS of the three unknown compounds were nearly identical, each showing a protonated molecular weight of 697 m/z. Each also showed neutral losses ((697 amu - 535 amu), and (535 amu - 373 amu)) of two hexoses (possibly glucose), yielding the fragment ion at 373 m/z. A loss of water (-18 amu) from this latter ion yielded a fragment ion at 355 m/z. The remaining fragment ion at 177 m/z does not correspond to any of the common hydroxycinnamic acids, and no additional information was obtained from the mass spectra.

However, when these compounds were analyzed by FTIR, the results confirmed the presence of glycosidic groups (ν_{O-H} 3350 cm$^{-1}$, and complex ν_{C-O} vibrations between 1400-1050 cm$^{-1}$), and of phenyl rings (1515 cm$^{-1}$), and carbonyl substituents (1700, 1626, 1599 cm$^{-1}$). The FTIR spectrum of the unknown compound B is shown in Fig. 7. Included in Fig. 7 is the FTIR spectrum of a mixture of hydroxycinnamates previously isolated from orange peel molasses (Manthey 2004). These latter compounds have been previously reported to constitute mainly ferulic and p-coumaric acid esters of neutral sugars and glucaric acids. The many similarities between the FTIR spectra of the unknown compound B and that of the mixture of hydroxycinnamates helps support a preliminary classification of these compounds as hydroxycinnamates, containing glycosidic side groups.

**Conclusion**

The results of these HPLC-PDA-MS analyses provide a preliminary characterization of the several main classes of...
These classes included ellagic acid and flavone glycosides, and complex hydroxycinnamates. The primary flavones in longan peel are quercetin and kaempferol. Further work is needed to complete the isolation and final identification of these compounds, particularly the set of unknown compounds believed to be hydroxycinnamates. These findings are important to the investigation of the chemical changes that occur in longan peel as a result of chilling injury.

Acknowledgement

Financial support from the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0011/2003) and USDA/ARS Citrus and Subtropical Products Laboratory, Winter Haven, Florida are acknowledged.

Literature Cited