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Screening and Identification of Antioxidants in Biological Samples Using High-Performance Liquid Chromatography−Mass Spectrometry and Its Application on *Salacca edulis* Reinw

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In this study, a new approach was developed for screening and identifying antioxidants in biological samples. The approach was based on significant decreases of the intensities of ion peaks obtained from high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) upon reaction with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radicals. HPLC-MS/MS was further applied to elucidate structures of antioxidant peaks characterized in a spiking test. The new approach could also be used to monitor the reactivity of antioxidants in biological sample with free radicals. The approach was successfully applied to the identification of antioxidants in salak (*Salacca edulis* Reinw), a tropical fruit that is reported to be a very good source of natural antioxidants, but it was still not clear which compounds were responsible for its antioxidant property. The antioxidants in salak were identified to be chlorogenic acid, (−)-epicatechin, and singly linked proanthocyanidins that mainly existed as dimers through hexamers of catechin or epicatechin. In salak, chlorogenic acid was identified to be an antioxidant of the slow reaction type as it reacted with free radicals much more slowly than either (−)-epicatechin or proanthocyanidins. The new approach was proved to be useful for the characterization and identification of antioxidants in biological samples as a mass detector combined with an HPLC separation system not only serves as an ideal tool to monitor free radical active components but also provides their possible chemical structures in a biological sample.

KEYWORDS: Salak; antioxidant; HPLC-MS; ABTS free radical; flavan-3-ols; chlorogenic acid

INTRODUCTION

The life expectancy of human beings has increased dramatically in the past century. However, as the average age of human beings has risen, the incidence of chronic age-related diseases such as cardiovascular disease, cancer, arthritis, Alzheimer's, Parkinson's, and macular degeneration have also increased. Clinical and epidemiological research support the hypothesis that consuming fruits and vegetables is beneficial to age-related diseases, several cancers, heart diseases, etc. (*1*−*5*). This could be partly attributed to those antioxidants contained in them (*1*, *2*, *6*−*11*) as fruits and vegetables are key sources of antioxidants in our diets. Antioxidant treatments are thought to offset radical damage to biomolecules, thereby slowing or delaying the onset of the diseases. As reviewed by Prior and Cao, a number of methods were established to evaluate the overall antioxidant capacity or total antioxidant activity (TAA) of biological samples (*12*). However, knowledge of the antioxidant compositions in fruits and vegetables will give us better information on their specific health effects and probably dosage of dietary intake.

The great complexity of biological samples has been a major obstacle to the identification of their antioxidant composition. Even though there have been numerous reports on this subject, only a small number of compounds were reported at one time in each individual study. High-performance liquid chromatography (HPLC) is the predominant method used to separate antioxidants such as phenolic compounds. Different detection systems have been used for the determination of separated compounds, for example, UV, electrochemical, and diode array detection (DAD) (*13*). UV detection is the most common. However, specificity for proanthocyanidins over other polyphenolic compounds is low due to the narrow range of UV absorption of these compounds (*14*). Postcolumn reaction of proanthocyanidins with dimethylaminocinnamaldehyde to produce a product with a maximum absorption at ∼640 nm was applied to increase sensitivity (*15*). Electrochemical detection of proanthocyanidins has also become more widespread due to the smaller number of electroactive substances compared to UV-absorbing compounds (*16*−*18*). In recent years, HPLC coupled with mass detection was widely used to identify unknown polyphenolics and proanthocyanidins in biological samples (*19*−*24*).

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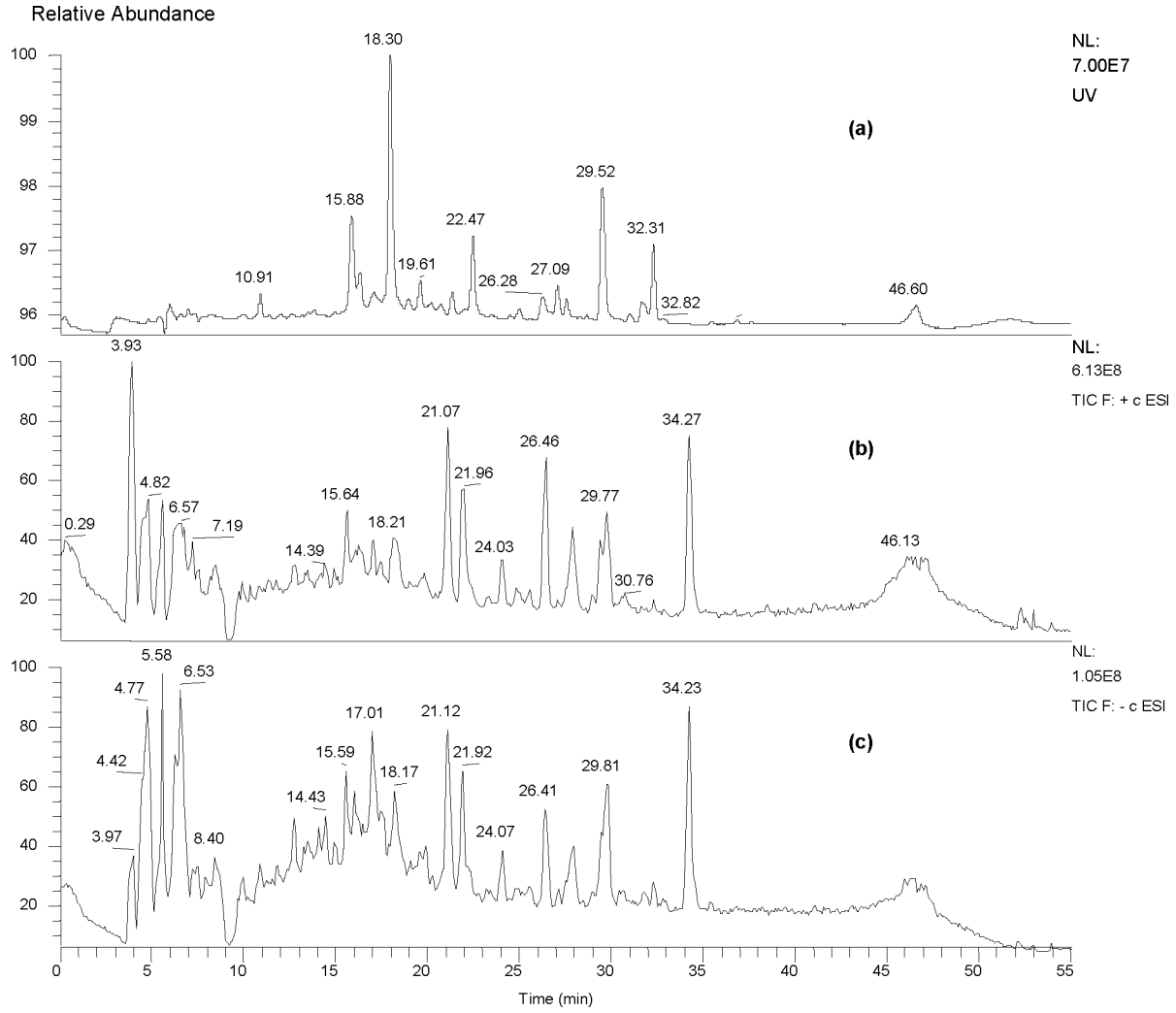

Figure 1. HPLC chromatograms of salak extract: (**a**) 280 nm; (**b**) TIC at positive mode; (**c**) TIC at negative mode.

Several HPLC methods, based on on-line detection of antioxidants by postcolumn reaction of eluates with free radicals, have been reported to be successfully applied to the identification and quantification of antioxidants in certain biological samples (*25*−*28*). Recently, we developed two approaches for the identification of major antioxidants in biological samples using a free radical spiking test using HPLC with UV or diode array detection (*29*, *30*). However, one disadvantage of these approaches is that they might not be applicable when antioxidant peaks are not sensitive to UV or diode array detectors. The main purpose of this study is to further improve the procedure for rapid screening and identification of free radical active components in a sample.

It is believed that the reaction between an antioxidant and a free radical results in the oxidation of the antioxidant. In the case of polyphenolic antioxidants, this is likely to involve the transfer of one or more hydrogen atoms from the antioxidant to the free radical. Once reacted, the molecular weight of products will most likely be different from that of unreacted antioxidants. On this basis, free radical active compounds could be monitored by observing the difference of peak intensity of individual ions between reactants and reaction products using a mass spectrometry detector. Even if the reactants and products are of the same molecular weights, by combining mass spectrometry with HPLC, they could still be characterized as they should be eluted at different retention times due to their structure difference. Therefore, HPLC coupled with a mass detector will be an ideal tool for characterizing free radical active compounds. Another advantage of this is that it is possible to characterize and identify those antioxidants that are not measurable because of their low absorbance and electrochemical detection properties. Previously developed methods (*29*−*31*) that were used to identify antioxidant peaks by spiking with free radicals followed by separation in HPLC-DAD were unable to identify some antioxidants in salak. These methods use methanol with aqueous sulfuric acid or formic acid as mobile phase. The failure to detect some antioxidants is most likely due to poor molar extinction coefficient of proanthocyanidins under the conditions specified.

Salak palm fruit belongs to the Arecaceae family and is indigenous throughout the Indo-Malaysian region. The fruit can be consumed fresh, candied, and canned when fully ripe. As reported previously, salak extract was an excellent free radical scavenger (*32*). However, it is still not clear which compounds are responsible for this scavenging ability. The newly developed approach was applied to the systematic characterization and identification of antioxidants in salak extract.

MATERIALS AND METHODS

Reagents. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), L-ascorbic acid, (−)-epicatechin, and potassium persulfate were from Sigma (St. Louis, MO);

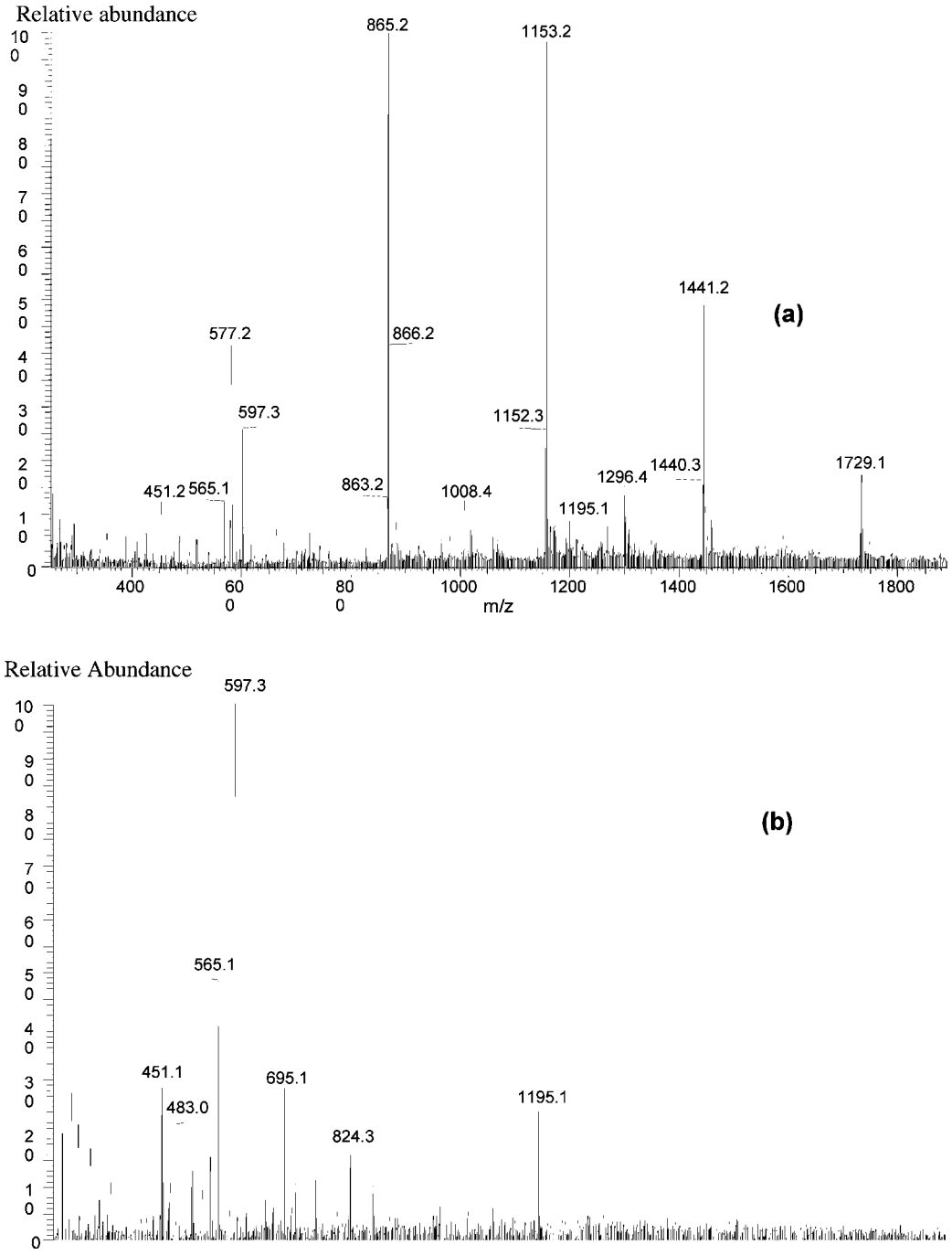

Figure 2. ESI-MS profiles of extract and reaction solution at stage 2 (10–20 min) under negative modes: (**a**) extract/water = 1:2; (**b**) extract/ABTS = 1:2.

(+)-catechin hydrate was from Aldrich Chemical Co. (Milwaukee, WI); ethanol, methanol, and hydrochloric acid were from Merck (Darmstadt, Germany).

Sample Preparation. Fresh salak with firm flesh and sweet taste was obtained from local markets. Salak was peeled, and seeds were removed from the flesh. Flesh was cut into small pieces and then homogenized. The homogenized flesh was extracted with 60% of aqueous ethanol for 1 h in the dark. The extract was centrifuged and filtered. The obtained solution was dried by rotary evaporation at 35 °C and redissolved in 20% aqueous methanol. The antioxidant capacity of the obtained solutions was measured, and it was found that the loss of total antioxidant activity (TAA) was <5% after evaporation and reconstitution. The obtained solution was immediately used for other assays or kept in the refrigerator for further experiments. The solution was filtered through a 0.45 μm membrane filter before HPLC-MS.

The TAA assay was carried out on the Ultraspec 3000 UV−vis spectrophotometer (Pharmacia Biotech Ltd., Cambridge, U.K.). The procedure was described by Leong and Shui with the results expressed as milligrams per 100 g of L-ascorbic acid equivalent antioxidant capacity (AEAC) (*32*). Similar procedures were used for measuring the reactivity of pure antioxidants with $ABTS^{\bullet+}$. The prepared $ABTS^{\bullet+}$ solution was diluted to obtain an initial absorbance of ~1.6 units at 730 nm (~1.0 mM) with a pH 4.5 HCl solution before use. Thirty microliters of 3.1 mM (−)-epicatechin and 30 mL of 3.3 mM chlorogenic acid were added to 3 mL of $ABTS^{\bullet+}$ solution, respectively. The changes of absorbance with time were recorded.

Characterization of Antioxidant Peaks in Salak by HPLC-MS. The apparatus was a Finnigan/MAT LCQ ion trap mass spectrometer (San Jose, CA) equipped with TSP spectra system, which includes a UV6000LP PDA detector, a P4000 quaternary pumo, and an AS3000 autosampler. The heated capillary and voltage were maintained at 250 °C and 4.5 kV, respectively. The full-scan mass spectra from *m/z* 50 to 2000 were recorded. For tandem mass spectrometry experiments, the collision energy was varied from 10 to 80%, and optimized collision

energy was chosen for individual compounds. Total ion chromatograms (TICs) were recorded in both positive and negative ionization models. Chromatographic separations were performed on a Shim-Pack VP-ODS column (250 × 4.6 mm i.d.) (Shimadzu, Kyoto, Japan) with a guard column (GVP-ODS, 10 × 4.6 mm i.d.) under the following elution conditions: flow rate = 500 μL min^{-1}; room temperature (~27 °C); solvent A, 0.1% formic acid in water; solvent B, methanol, starting from 20 to 50% B in 20 min, from 50 to 90% B in 20 min, and maintained for 10 min, from 90 to 20% B in 5 min, and finally isocratically at 20% for 10 min to recondition the column. Mass spectra were recorded over the 45 min period.

One milliliter of extract and 2 mL of ABTS$^{\bullet+}$ were mixed to react for 1 h and then passed through 0.45 μm filter and injected for HPLC-MS assay. Water (2 mL) was added to the extract to provide an unreacted control sample.

RESULTS AND DISCUSSION

Free Radical Active Ion Peaks in Salak Extract. Figure 1 shows the UV chromatogram at 280 nm and total ion chromatograms (TIC) in the positive and negative modes. It is interesting to find that both the TIC in the positive ion mode and that in the negative ion mode by mass detector provided more peaks than those observed by UV detection at 280 nm. In addition, the UV data did not clearly indicate the presence of monomer through hexamers in the salak extract (**Figure 1a**). Extracted ion chromatograms of any interesting ions will also provide additional information regarding the compound. For these reasons, this method provides absolute advantages over any other common detection systems.

To investigate which peaks in salak extract belong to antioxidants, the intensities of individual ion peaks were compared between that of the control and reaction products of free radical with salak extract. As so many ion peaks existed in TICs of salak extract, rough comparisons at different elution periods on ion peaks and their intensities between control and reaction solution were carried out. It was found that most compounds in salak extract were eluted before 40 min; therefore, ion peaks of both reaction solution and control were divided into four elution stages with an interval of 10 min. The mass spectrometry profile of each stage in the positive and negative ion modes was investigated.

Of the four stages investigated, averaged mass spectra at retention time from 10 to 20 min show that intensities of many ion peaks at stage 2 disappear or are significantly reduced after reacting with free radicals. **Figure 2** shows the mass spectrum profiles of control and reaction solution at stage 2 in the negative ion mode.

Peaks eluted are summarized in **Table 1**, where their retention times, *m*/*z* of ions, and intensities before and after reaction with ABTS$^{\bullet+}$ are given. Antioxidants that reacted with ABTS$^{\bullet+}$ showed lower intensities and are indicated in the last column to be reactive toward ABTS$^{\bullet+}$. There were no significant changes for intensities of those ion peaks that were eluted before 10 min. Similarly, there was no significant decrease of intensities of ion peaks eluted after 20 min except for (−)-epicatechin, which has the retention time of 20.4 min (**Table 1**). Although intensities of some peaks at stage 3 (from 20 to 30 min) and stage 4 (from 30 to 40 min) were slightly reduced when mixed with a high concentration of ABTS$^{\bullet+}$ (**Table 1**), they are tentatively not considered as antioxidants because their intensities are not reduced when mixed with ABTS$^{\bullet+}$ at lower concentrations. This will be discussed further below.

Those peaks that had retention times between 10 and 20 min were identified as antioxidant-related peaks as their intensities were significantly reduced after reacting with ABTS$^{\bullet+}$. HPLC-MS/MS was further applied to elucidate the possible structures of corresponding antioxidants.

Table 1. Ion Peak Intensities of Extract and Its Reaction Solution with ABTS$^{\bullet+}$

		intensities of ion peaks[a]		
m/*z**	t_R (min)	control (extract/water = 1:2)	reaction product 1 (extract/ABTS$^{\bullet+}$ = 1:2)	free radical reactivity, ABTS$^{\bullet+}$
577 (–)	14.83	7.4 ± 0.3E6	<1E5	yes
	15.99	4.5 ± 0.2E6	<1E5	yes
865 (–)	13.15	8.3 ± 0.4E6	<1E5	yes
	14.52	2.5 ± 0.2E6	<1E5	yes
	15.37	2.5 ± 0.2E6	<1E5	yes
	17.42	8.4 ± 0.3E6	<1E5	yes
	18.03	4.2 ± 0.2E6	<1E5	yes
1153 (–)	11.28	3.7 ± 0.3E6	<1E5	yes
	13.17	1.4 ± 0.1E6	<1E5	yes
	13.60	2.6 ± 0.2E6	<1E5	yes
	14.80	2.5 ± 0.1E6	<1E5	yes
	17.50	4.9 ± 0.3E6	<1E5	yes
	30.12	1.6 ± 0.2E6	1.3 ± 0.2E6	no
1441 (–)	12.71	1.0 ± 0.1E6	<1E5	yes
	16.53	2.2 ± 0.2E6	<1E5	yes
	17.77	1.4 ± 0.1E6	<1E5	yes
1729 (–)	17.42	0.5 ± 0.1E6	<1E5	yes
453 (+)	16.84	5.5 ± 0.2E6	5.1 ± 0.5E6	no
355 (+)	18.43	3.8 ± 0.1E6	1.3 ± 0.1E6	yes
289 (–)	20.4	1.3 ± 0.3E6	<1E5	yes
464 (+)	21.2	5.4 ± 0.3E7	6.1 ± 0.7E6	no
482 (–)	22.3	6.5 ± 0.3E6	5.3 ± 0.6E6	no
393	24.4	8.5 ± 0.3E6	7.3 ± 0.4E6	no
	30.1	1.5 ± 0.3E7	1.2 ± 0.2E6	no
419	22.5	0.9 ± 0.3E6	0.9 ± 0.3E6	no
	28.0	3.0 ± 0.3E6	2.7 ± 0.3E6	no
	29.6	1.2 ± 0.3E6	1.1 ± 0.3E6	no
444	26.7	4.0 ± 0.3E6	4.2 ± 0.3E6	no
875	26.7	3.4 ± 0.3E7	3.1 ± 0.3E7	no
471	26.7	8.1 ± 0.3E6	7.7 ± 0.3E6	no
739	34.5	6.9 ± 0.3E6	5.2 ± 0.3E6	no
723	34.4	1.3 ± 0.3E7	1.1 ± 0.3E6	no
721	34.4	4.0 ± 0.3E6	3.9 ± 0.3E6	no
745	34.5	5.1 ± 0.3E6	5.2 ± 0.3E6	no
699	34.5	7.2 ± 0.3E6	6.3 ± 0.3E6	no

[a] $n \pm$ SD = 3, each performed in triplicate.

The extracted ion chromatograms of antioxidants are shown in **Figure 3**. Although **Table 2** shows profiles of antioxidant peaks in salak extract, other ions (e.g., ions at *m*/*z* 289 with a retention time of 3.98 min and at *m*/*z* 1153 with a retention time of 30.08 min), which had the same signal of *m*/*z* as some of the antioxidant peaks, were not listed in the table as their intensities were not reduced and therefore were not characterized as antioxidants.

Identification of Antioxidants by HPLC-MS/MS. Initial TICs of salak juice/extract, which is obtained by HPLC-ESI-MS in both the positive and negative ion modes, indicated that antioxidant peaks have molecular masses of 578, 866, 1154, 1442, and 1730, respectively. They have molecular masses of [290 × *n* − (*n* − 1) × 2], which indicated that they were proanthocyanidins formed by catechin and epicatechin units and had been proven to be excellent free radical scavengers (*33*, *34*). The interflavanic linkage usually occurs between C4 and C6 or between C4 and C8 (**Figure 4**).

To confirm the existence of proanthocyanidins, tandem mass spectrometry of those antioxidant peaks was further investigated. The CID spectra of these compounds are given in **Table 2**. The main fragment ions of parent ions at *m*/*z* 579 were *m*/*z* 427 [M + H − 152]$^+$ from retro-Diels−Alder fission (RDA-F) of the heterocyclic rings, *m*/*z* 409 [M + H − 170]$^+$ from RDA-F of the heterocyclic rings and loss of water, and *m*/*z* 291 [M + H − 288]$^+$ from interflavanic bond cleavage. Similarly, fragment

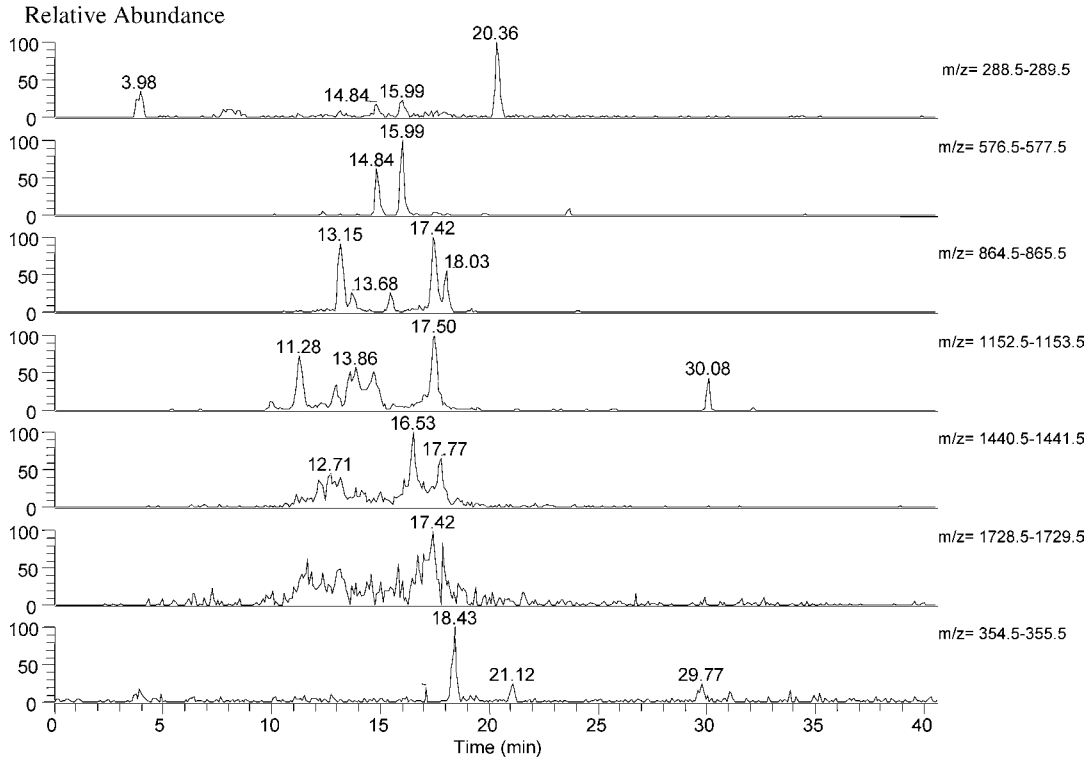

Figure 3. Extracted ion chromatograms for possible antioxidant ion peaks.

Table 2. Positive and Negative Ions and Their Corresponding CID Ions of Antioxidants

		ESI MS *m*/*z*		
t_R (min)	mode	$[M \pm H]^{\pm}$	$[M + Na]^+$	major CID ion peaks of $[M \pm H]^{\pm}$
14.83, 15.99	+	579	601	427, 409, 291, 289, 453
	−	577		not investigated
13.15, 14.52, 15.37, 17.42,18.03	+	867	889	715, 697, 579, 577, 427, 409, etc.
	−	865		not investigated
11.28, 13.17, 13.60, 14.80, 17.50	+	1155	1177	1003, 985, 867, 865, 715, 697, 579, 577, 427, 409, etc.
	−	1153		not investigated
12.71, 16.53, 17.77	+	1443	1465	1291, 1273, 1155, 1153, 867, 865, 579, 577, etc.
	−	1441		not investigated
17.42	+	1731		1579, 1561, 1443, 1441, 1155, 1153, 867, 865, etc.
	−	1729		not investigated
18.42	+	355		163
	−	353		191, 179
20.4	+	291		123, 139, 165, etc.
	−	289		245, 205, 179, etc.

ions of *m*/*z* 867 included those at *m*/*z* 579 $[M + H - 288]^+$ from interflavanic bond cleavage and *m*/*z* 715 $[M + H - 152]^+$ from RDA-F of the heterocyclic. For compounds with molecular masses of 1154, 1442, and 1730, their CID spectra underwent fragmentation similar to those with molecular masses of 578 and 866. Their fragment patterns are the same as proanthocyanidins in star fruit and pyconogenol (*29*). Therefore, these compounds were assigned as singly linked proanthocyanidins existing as dimers through hexamers, respectively.

As shown in **Figure 2a**, another two peaks with ions *m*/*z* 1008.4 and 1296.2, respectively, were also significantly reduced after reaction with $ABTS^{\bullet+}$. They might arise from doubly charged heptamer $[290 \times 7 - (7 - 1) \times 2 - 2]^{2-}$ and nanomer $[290 \times 9 - (9 - 1) \times 2 - 2]^{2-}$, respectively. No significant peaks were observed in their extracted chromatograms, probably due to low concentrations in the extract (data not shown). Because of the limitation of instrumentation on mass range, that is, 50−2000 Da, their parent ion could not be detected. Their

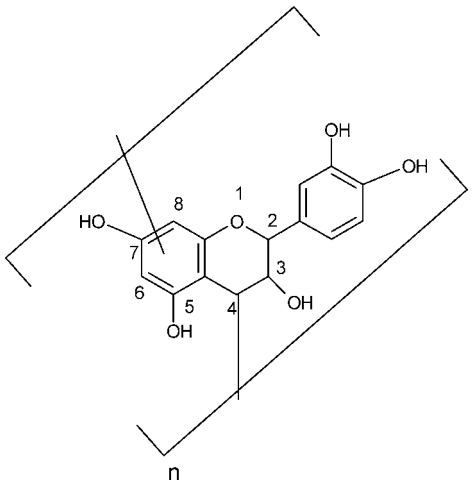

Figure 4. Chemical structures of proanthocyanidins in salak extract (n = 1–6).

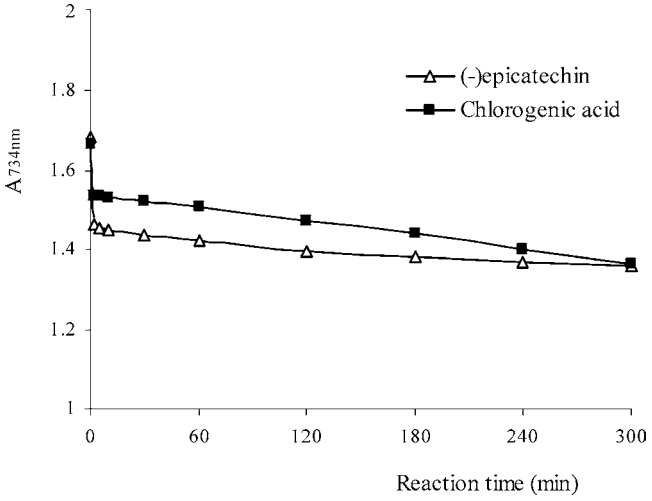

Figure 5. Kinetics of $ABTS^{\bullet+}$ with (–)-epicatechin and chlorogenic acid. Reaction conditions: room temperature; 30 μL addition of 3.1 mM of (–)-epicatechin, and 30 μL addition of 3.3 mM chlorogenic acid into 3 mL of 1.0 mM ABTS solution.

charge states were also not observed by further zoom scan, probably due to too low contents in sample.

Other than proanthocyanidins identified by tandem mass spectrometry, the intensities of another two peaks were also significantly reduced after reaction with $ABTS^{\bullet+}$ for 1 h. One peak had the retention time of 18.43 min (**Figure 3**) and ions at *m*/*z* 355 $[M + H]^+$ and 353 $[M - H]^-$, respectively. Fragment ions *m*/*z* 179 and 191 from $[M - H]^-$ at *m*/*z* 353 indicated that the peak arose from the ester of caffeic acid with quinic acid. The peak was confirmed to be chlorogenic acid by spiking the extract with a standard. Another peak was found to be (−)-epicatechin by spiking test and comparing its CID spectra with standard.

While using these ionization techniques in HPLC-MS, some precautions were taken. Although $[M + H]^+$ or $[M - H]^-$ predominates in most compounds, mass spectrometry from ESI technique may include adduct ion peaks such as $[M + Na]^+$, $[M + K]^+$, $[M + M + Na]^+$, $[M + M + H]^+$, $[M + M - H]^-$, and multiple charged ions. Therefore, much attention was given when any of those additional peaks appeared with high relative abundance together with protonated or deprotonated ions. In such cases, predominant ion peak(s) should be used for the characterization of free radical active components.

Reactivity of Antioxidants with Free Radicals. Different varieties of antioxidants may exhibit different reaction types with free radicals because of their chemical structure differences. An antioxidant that reacts with free radicals rapidly is considered to be more efficient compared to those which react slowly with free radicals. For example, after reacting with high concentration of $ABTS^{\bullet+}$ for 1 h, HPLC-MS analysis showed that ~30% of chlorogenic acid in the extract is still not consumed. This indicated that the reaction between chlorogenic acid and $ABTS^{\bullet+}$ is slow, whereas (−)-epicatechin is almost fully consumed after reacting with $ABTS^{\bullet+}$ for 1 h. Similarly, proanthocyanidins also reacted with $ABTS^{\bullet+}$ rapidly. The results were consistent with kinetics of $ABTS^{\bullet+}$ with (−)-epicatechin and chlorogenic acid (**Figure 5**), which shows that $ABTS^{\bullet+}$ reacts much more quickly with (−)-epicatechin than with chlorogenic acid at initial reaction stage.

Although some peaks eluted at stages 3 and 4 were slightly reduced when mixed with a high concentration of $ABTS^{\bullet+}$, they were tentatively not considered as antioxidants because they were not efficient free radical scavengers. This is true even when compared with chlorogenic acid, which is considered to be a less effective antioxidant than (−)-epicatechin and proanthocyanidins. Similar results were obtained when salak extract was spiked with DPPH free radicals.

Conclusion. A new approach based on HPLC-MS screening of free radical active components in biological samples and $HPLC/MS^n$ structural elucidation of active components was successfully applied to the identification of the major antioxidants in salak. Chlorogenic acid, (−)-epicatechin, and proanthocyanidins existing as dimers through hexamers were reported for the first time in salak and preliminarily considered as major antioxidants in salak. The new approach was successfully applied to the identification of antioxidants in ciku king (*35*) and ulam raja (paper in preparation) and can also be widely used for the identification of antioxidants in other biological samples.

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