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# Short Communication

# Characterization of flavonoids by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Thermospray LC–MS–MS was used to study a catechin mixture extracted from the tea plant *Camellia sinensis*. Four major catechins, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin 3-O-gallate, and (−)-epigallocatechin 3-O-gallate were quickly recognized in the mixture by their $[M + H]^+$ ions. Collission-induced dissociation (CID) spectra of the $[M + H]^+$ ions gave simple fragmentation patterns, which permitted characterization of the substituents and the ring structures of the molecules. Several representative flavonoids and their glycosides were also studied by thermospray LC–MS–MS. Their CID spectra showed three types of ring cleavages in the pyran ring of the flavonoids, and differentiation among flavanone, flavone, and flavonol was made. LC–MS–MS appears to be the method of choice for the analysis of plant flavonoids including their glycosides.

## INTRODUCTION

The history of tea in China started as early as 5000 years ago, when it was discovered as a herbal medicine, and some significant pharmacological and health effects of the tea plant, *Camellia sinensis*, have gained support from scientific research in recent years. Catechins, the main flavonoid constituents of *Camellia sinensis*, have been shown to be inhibitory against mutagenic and tumorigenic activities [1-5]. (−)-Epigallocatechin 3-O-gallate, the most abundant catechin in the plant, was shown to have a radioprotective effect when it was administered to mice [6]. Catechins have also been known to have broad antibacterial and antiviral activities against herpes simplex, leukemia, influenza and cytomegaloviruses. Two catechins, (−)-epigallocatechin 3-O-gallate and (−)-epicatechin 3-O-gallate, were found to inhibit HIV reverse transcriptase and cellular DNA and RNA polymerases [7]. Although

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the mechanism of the pharmacological effects of catechins remains unclear, the strong antioxidant activity of catechins as radical scavengers might play some role [8]. Several polyphenols from plants, such as methylgallate [9], 5,6,7-trihydroxyflavone [10], oxidized polymeric compounds of caffeic acid [11], and tannic acid [12], have also been reported to have antiviral activities.

In connection with our interest in the study of structure and biological activity relationship of flavonoids [13], we have explored an effective means of mass spectrometric identification of plant flavonoids by LC–MS–MS. Catechin mixtures extracted from *Camellia sinensis* and several other plant flavonoids (Fig. 1) were studied by thermospray LC–MS–MS, and we found that this technique can be used effectively for the identification of catechins and other flavonoids in complex mixtures.

## EXPERIMENTAL

### *Materials*

A catechin mixture was supplied by Zejiang Agricultural University, Hangzhou, China. The mixture was obtained by extraction of green tea leaves of *Camellia sinensis* briefly as follows [14]: the dried tea leaves were extracted three times with approximately 10 volumes of hot water. Catechins were precipitated with $CaCO_3$ solution to separate them from the caffeine, redissolved in acid medium, and re-extracted into ethyl acetate. Evaporation of the ethyl acetate gave light brown powders, which consisted mostly of catechins. Pure flavonoids were obtained from Aldrich (Milwaukee, WI, USA).

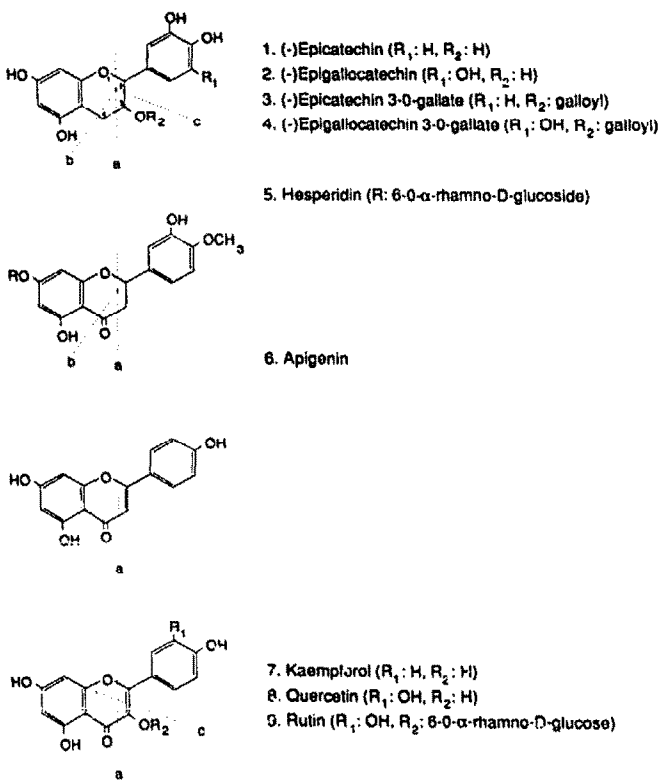


Fig. 1. Structures of catechins and flavonoids.

### *LC–MS–MS*

Mass spectra were obtained with a Finnigan MAT TSQ 70 mass spectrometer interfaced to a Waters 600-MS HPLC system. For HPLC a methanol–water (30:70) mixture containing 0.05% trifluoroacetic acid was used at a flow-rate of 1 ml $min^{-1}$. A Waters reversed-phase $C_{18}$ column, 25 cm × 2.0 mm I.D. packed with 5-$\mu$m particles, was used. The effluent from the column was passed into the Finnigan MAT thermospray LC–MS interface. Collision-induced dissociation (CID) spectra were obtained at 20–70 eV collision energy using argon at 2–4 mTorr (1 Torr = 133.322 Pa) in the non-linear collision cell.

## RESULTS

### *Thermospray LC–MS and CID spectra of tea catechin mixture*

The mass chromatogram of the thermospray LC–MS obtained with a 30-$\mu$l injection of 1 mg/ml of the catechin mixture is shown in Fig. 2. The reconstructed total ion chromatogram, bottom trace of Fig. 2, showed only four distinct peaks. The mass spectra of the peaks (Table I) all gave abundant $[M + H]^+$ ions consistent with the expected four major tea catechins [15], (−)-epicatechin ($m/z$ 291), (−)-epigallocatechin ($m/z$ 307), (−)-epicatechin 3-O-gallate ($m/z$ 443), and (−)-epigallocatechin 3-O-gallate ($m/z$ 459). As clearly demonstrated by the mass chromatograms in Fig. 2, the thermospray LC–MS provides mass spectrometric identification of the four major catechins in the plant. In addition to the $[M + H]^+$ ions, the presence of gallic esters in (−)-epicatechin 3-O-gallate (**3**) and (−)-epigallocatechin 3-O-gallate (**4**) are indicated in the LC–MS spectra by the ions $m/z$ 275 and 291 derived from the loss of 168 u from the corresponding $[M + H]^+$

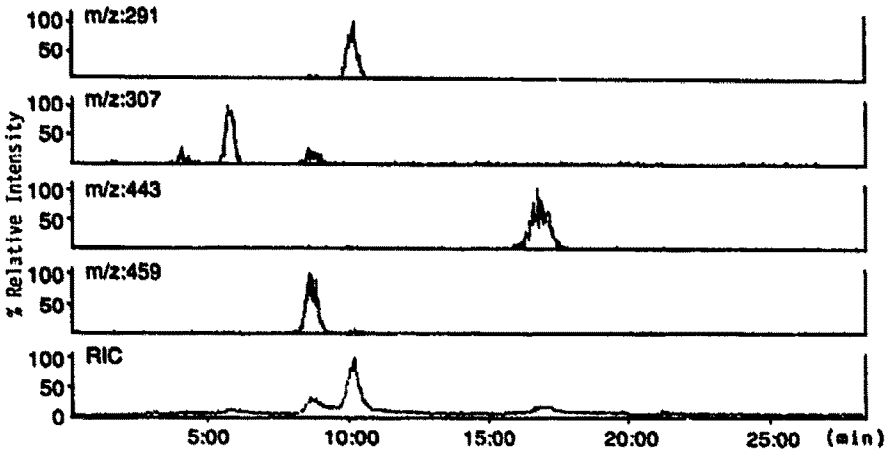


Fig. 2. Mass chromatograms of a catechin mixture from *Camellia sinensis* analyzed by thermospray LC–MS. RIC = reconstructed total ion chromatogram.

ions. Furthermore, in the CID spectra ions of *m/z* 153 and 151 derived from galloyl moiety as well as the ions *m/z* 273, 289 ([M + H − 170]) derived, respectively, from the loss of gallic acid (170 u) from the corresponding $[M + H]^+$ ions (Table I) also indicated the presence of gallic esters. We have also analyzed the mixture with particle-beam LC–MS, but the results were less informative, because two of the catechins, (−)-epicatechin 3-O-gallate (**3**) and (−)-epigallocatechin 3-O-gallate (**4**), failed to give molecular ions, only fragmentation ions (results not shown).

In order to obtain additional structural information from fragmentation ions, CID spectra of the above $[M + H]^+$ ions were studied. The CID spectra of catechins consist of three types of cleavages, *a*, *b* and *c*, at the dihydropyran rings of catechins, and elimination ions of the substituent at the 3-position of the ring. Fig. 3 is an example of the CID spectrum for the $[M + H]^+$ (*m/z* 443) ion of (−)-

TABLE I

THERMOSPRAY MS AND CID SPECTRA OF CATECHINS AND FLAVONOIDS

| Compound | Thermospray MS *m/z* (rel.%)[a] | | CID spectra *m/z* (rel.%)[a] | | | |
|---|---|---|---|---|---|---|
| | $[M + H]^+$ | [others] | [A] | [B] | [C] | [others] |
| 1 | *291*(100) | 275(6) | 139(100) | 165(10) | 123(58) | 207(18), 147(13) |
| 2 | *307*(100) | | 139(100) | 181(10) | 139(100) | 223(4), 163(7) |
| 3 | *443*(100) | 291(40), 275(100), 273(24) | 139(24) | 165(5) | 123(100) | 291(47), 273(18), 153(14), 151(22) |
| 4 | *459*(40) | 307(13), 291(100), 285(25) | 139(100) | 181(5) | 139(100) | 307(17), 289(34), 153(14), 151(18) |
| 5 | 611(10) | 465(8) | | | | |
| | | *303*(100) | 153(100) | 177(68) | – | |
| | | | 149(19) | | | |
| 6 | *271*(100) | | 153(89) | – | – | |
| | | | 119(100) | | | |
| 7 | *287*(100) | | 153(100) | – | 121(70) | |
| 8 | *303*(100) | | 153(100) | – | 137(60) | |
| 9 | *611*(8) | 465(3) | – | – | – | 465(27), 303(100), 165(7), 147(24), 129(45) |
| | | *303*(100) | 153(100) | – | 137(70) | |

[a] CID spectra were obtained with the italicized MS ions.

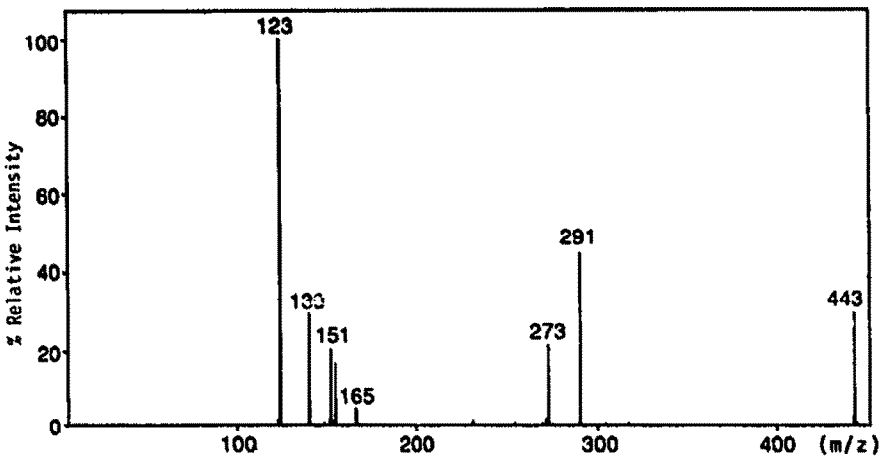


Fig. 3. CID spectrum of $[M + H]^+$ ($m/z$ 443) of (−)-epicatechin 3-O-gallate (**3**). The CID spectrum was obtained using a collision energy of 21 eV.

epicatechin 3-O-gallate **3**. The spectrum is simple yet informative for the structure, and the assignment of the ions can be made as follows: $m/z$ 443 ($[MH^+]$), 291 ($[MH^+ - R_2 + H]$), 273 ($[MH^+ - R_2OH]$), 165 (cleavage $b - R_2 + H$), 153 ($[R_2^+]$), 151 ($[R_2^+ - H_2]$), 139 (cleavage $a$), and 123 (cleavage $c$). The CID spectra of other catechins are included in Table I.

*Thermospray MS and CID spectra of some representative flavonoids*

In addition to catechins, we also studied thermospray MS and CID spectra of some representative flavonoids: flavanone, hesperidin **5**; flavone, apigenin **6**; and flavonols, kaempferol **7**, quercetin **8**, and rutin **9** (Fig. 1). The thermospray MS of non-glycosylated flavonoids **6**, **7**, and **8** all gave single $[M + H]^+$ peaks with no fragmentation. The thermospray MS of two flavonoid O-glycosides, hesperidin **5** and rutin **9** gave simple three ion spectra of $m/z$ 611, 465, and 303, which clearly represent the ions of $[M + H]^+$, [M − rhamnosyl], and [M − rhamnoglucosyl moieties]; thus, both aglycone and glycosyl substituents in the molecules can be recognized. However, further characterization of the aglycone and glycosyl components was achieved by the study of their CID spectra. As shown by the spectra in Table I and the structures in Fig. 1, all catechin and flavonoid molecules undergo facile cleavages $a$ and produce $m/z$ 139 with catechins **1**–**4** and $m/z$ 153 for flavonoids **5**–**9**. The facile cleavage $a$ in catechins or flavonoids can be attributed to the formation of stable arylcarbonium and or arylcarbonyl ions, respectively. The cleavage $a$ ions, such as $m/z$ 139 or 153, in the CID spectrum generate the diagnostic ions for the identification of catechin or flavonoid molecules in complex mixtures. Cleavages $b$ were observed with catechins **1**–**4** and flavanone **5**, but not with flavone **6** or flavonols **7**–**9**. The absence of cleavage $b$ in flavone or flavonol may due to the presence of the $\alpha,\beta$-unsaturated ketone in the pyran ring resulting in stabilization of the ring bond between C-4 and C-5 by the extension of arylic conjugation between the two aromatic rings. Cleavage $c$, the cleavage of the bond between C-2 and C-3 of the pyran ring, is only observed with catechins **1**–**4** and flavonol **7**–**9**, which have O-substitutions at the C-3 position. Therefore, differentiation among catechin, flavanone, flavone, and flavonol can be made by CID spectra. For a glycosyl flavonoid, rutin **9** for example, CID spectra can be obtained with either the $[M + H]^+$ $m/z$ 611 or the ion of aglycone $m/z$ 303. From the CID spectrum of $m/z$ 303, the aglycone of the flavonoid can be characterized as a flavonol by the cleavage $a$ ($m/z$ 153) and cleavage $c$ ($m/z$ 137), and additional fragmentation ions indicative of the disaccharide moieties are obtained by the CID spectrum of $m/z$ 611 (Table I).

## DISCUSSION

Comprehensive information on electron impact (EI) MS fragmentation patterns is available for the major classes of aglycone flavonoids; only when the

individual flavonoids are subjected to EI-MS analysis, can their fragmentation be assigned and distinguished [16–20]. However, flavonoids are usually present as non-volatile mixtures in plants, and their analysis usually must be carried out by GC–MS with their trimethylsilyl or permethyl derivatives [21–24]. LC–MS with moving belt has been used to study flavonoids under both EI and chemical ionization (CI) conditions. Under the CI conditions, the spectra provide only $[M + H]^+$ ions for molecular mass information with little fragmentation; on the other hand, EI–MS has been hampered by difficulties with high background contribution and notable diminution of the molecular ion, especially with highly polar and non-volatile compounds [25]. Fast atom bombardment MS has been used to study the glycosidic linkages in glycosyl flavonoids, but it has provided little information on the aglycone structures [26]. Therefore, the thermospray LC–MS–MS we have shown appears to be the method of choice for the study of flavonoids, including their glycosides, as complex mixtures that exist in the plant kingdom.

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