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Rapid Analysis of Theaflavins by High Performance Liquid Chromatography / Atmospheric Pressure Chemical Ionization-Mass Spectrometry

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ABSTRACT

A 10 cm x 4.6 mm i.d. SilicaROD RP-18 HPLC column coupled with isocratic elution with a solvent system of acetonitrile-2% acetic acid or acetonitrile-1% citric acid were used to analyze purchased theaflavin mixtures and theaflavin extracts from black tea. Individual theaflavins were well separated and the analysis of theaflavins was achieved in 10 min using this method. HPLC coupled with Atmospheric Pressure Chemical Ionization-Mass Spectrometry (APCI-MS) in the positive ion mode was used to identify individual theaflavins. Quasi-molecular ions and many structural characteristic fragment ions were observed in the mass spectra. Possible fragmentation pathways from the protonated theaflavins were also proposed.

Key words: **theaflavins, Atmospheric Pressure Chemical Ionization, APCI, liquid chromatography / mass spectrometry, LC/MS.**

INTRODUCTION

Theaflavins are a group of seven-membered ring compounds derived from catechins or their gallates during black tea manufacturing. Theaflavins are bright-red in color and contribute substantially to the color and taste of black tea beverage although they are present only at a level of 1.5-2.5% in the dry leaves [1]. It has been shown that theaflavins possess antioxidant activities which are related to lipoxygenase inhibition activity [2], 1,1-diphenyl-2-picrylhydrazyl radical scavenging ability [3], and superoxide radical scavenging ability [3]. These antioxidant properties of theaflavins imply that theaflavins may be physiologically beneficial to human body.

Spectrophotometry and high performance liquid chromatography (HPLC) have been commonly used to analyze theaflavins [4]. The former was used for the analysis of total theaflavins, while the latter was used to identify individual theaflavins. HPLC is superior to spectrophotometry in quantification accuracy, and can be used for analysis of individual theaflavin. Various methods based on

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HPLC have been developed for the separation and analysis of theaflavins recently.

In a reversed-phase HPLC column, the mixture of four main theaflavins, the structures of which are shown in Figure 4, was separated in an order of theaflavin, theaflavin-3-monogallate, theaflavin-3'-monogallate, and theaflavin-3,3'-digallate [4-7]. A mobile phase of acetonitrile-acetic acid solution pumped with a gradient elution was used for the reversed-phase column. However, it could not achieve a base-line separation of theaflavin-3'-monogallate and theaflavin-3,3'-digallate [5,6]. Improvements in resolution and peak shape were achieved using citrate buffer-acetonitrile as a mobile phase [6]. Citric acid as a chelating agent reduced secondary retention through removal or masking of surface metals.

Although the analysis of theaflavins can be accomplished using a reversed-phase HPLC column coupled with gradient elution with a mobile phase of acetonitrile-acetic acid solution or acetonitrile-citric acid solution, current HPLC procedure is still time-consuming. It usually takes more than 40 min to complete one run. Besides, the theaflavin standards are not commercially available. The use of UV or photodiode-array detectors is still not adequate for an unequivocal identification without standards. It is, therefore, necessary to search for a rapid HPLC method and more definite identification .

In the present paper, we present an HPLC method capable of rapidly analyzing theaflavins in 10 min using a SilicaROD RP-18 column and an isocratic elution with a mobile phase of acetonitrile-2% acetic acid or acetonitrile-1% citric acid. In order to obtain more reliable identification, we used an Atmospheric Pressure Chemical Ionization-Mass Spectrometry (APCI-MS), in which a quasi-molecular ion $[M+H]^+$ and many structural characteristic fragment ions were generated and appeared in the mass spectra.

MATERIALS AND METHODS

I. *Materials*

Black tea was obtained from Taiwan Tea Experiment Station (Yangmei, Taiwan). A theaflavin mixture containing 80% theaflavins was purchased from Sigma Chemical Co. (St. Louis, MO)

II. *Preparation of Theaflavin Extracts from Black Tea*

Black tea powder (3.0 g) in 100 ml of boiling water was refluxed for 30 min. After filtration, the

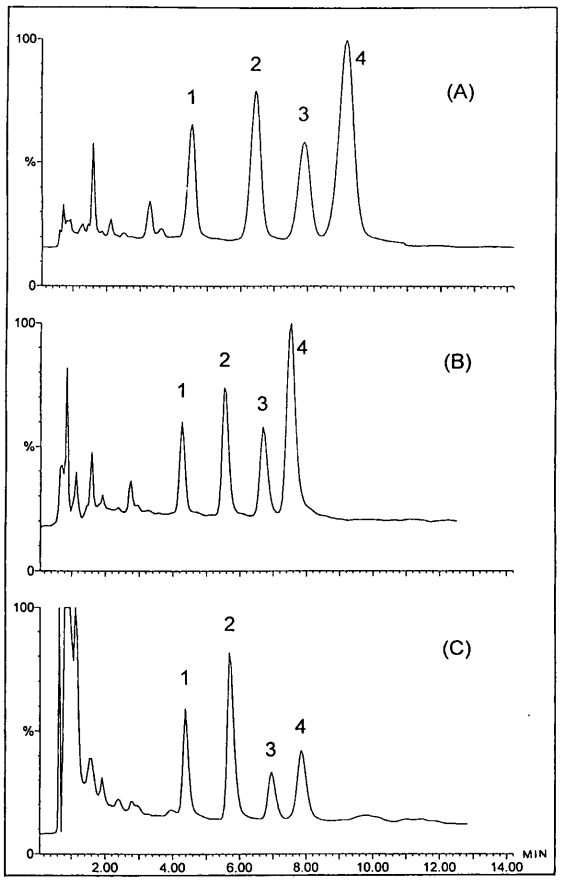


Figure 1. Reversed-phase HPLC chromatograms of theaflavins from purchased theaflavin mixture (A and B) or extracted from black tea (C). Analyses were performed with a 10 cm x 4.6 mm i.d. SilicaROD RP-18 column using isocratic elution with acetonitrile-1% citric acid adjusted to pH 2.8 with 1N NaOH (22:78, v/v) (A), or acetonitrile-2% acetic acid (21:79, v/v) (B and C) at a flow rate of 2.5 ml/min. Theaflavins were monitored at 280 nm. Peak 1: theaflavin, peak 2: theaflavin-3-monogallate, peak 3: theaflavin-3'-monogallage, peak 4: theaflavin-3-3'-digallate.

filtrates were extracted with 3 x 50 ml of ethyl acetate. The combined ethyl acetate phases were washed with a 2.5% sodium bicarbonate solution (3 x 30 ml). The ethyl acetate layer was then evaporated to dryness to obtain crude theaflavins.

III. *HPLC and LC/MS Analysis*

HPLC analysis was performed using a Jasco PU-980 Intelligent HPLC pump coupled with an Applied Biosystems 783A Programmable Absorbance Detector. A 10 cm x 4.6 mm i.d. SilicaROD RP-18 column (Merck, Darmstadt, Germany) was used. Elution was carried out at a flow rate of 2.5 ml/min using a solvent system of acetonitrile-2% acetic acid (21:79, v/v) or acetonitrile-1% citric acid adjusted to pH 2.8 with 1N NaOH (22:78, v/v) isocratically. The eluate was monitored at 280 nm. For the LC/MS analysis, a solvent system of acetonitrile-2% acetic acid (21:79, v/v) at a flow rate of 1.7 ml/min was used. The eulate was also monitored at 280 nm prior to introducing to MS. Mass analysis was performed on a Micromass Platform system (Micromass Co. Manchester, UK) equipped with a Digital Celebris GL 5120 computer for data analysis. Positive ion mass spectra were obtained by using a heated nebulizer-APCI interface. The ion source temperature was set at 150°C and the sample cone voltage was 10 or 40 V.

RESULTS AND DISCUSSION

I. *HPLC Analysis*

Figure 1 presents the HPLC chromatograms

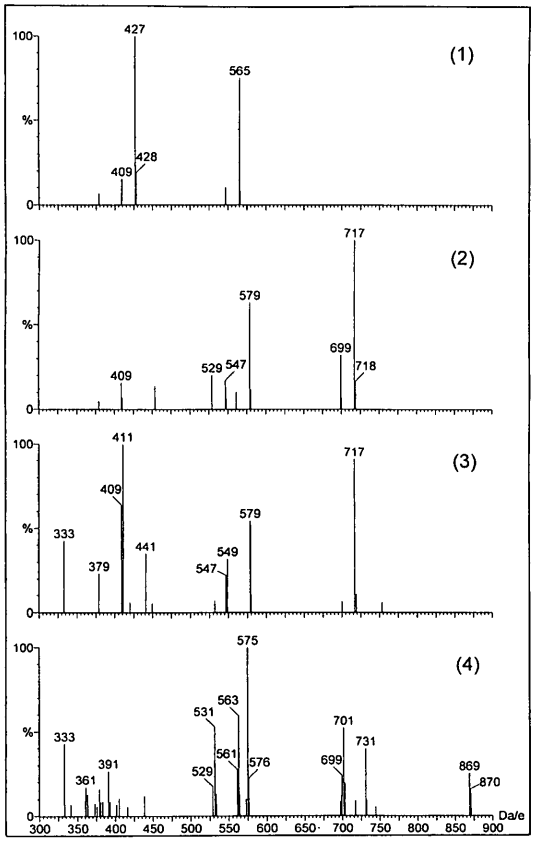


Figure 2. Positive ion mass spectra of (1) theaflavin, (2) theaflavin-3-monogallate, (3) theaflavin-3'-monogallate, and (4) theaflavin-3,3'-digallate separated by HPLC. A sample cone voltage of 40 V was used for the APCI-MS analysis.

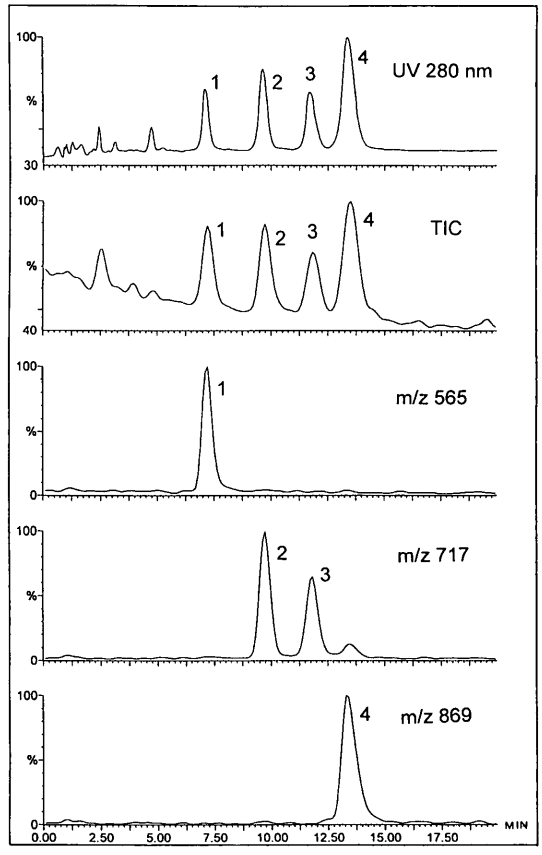
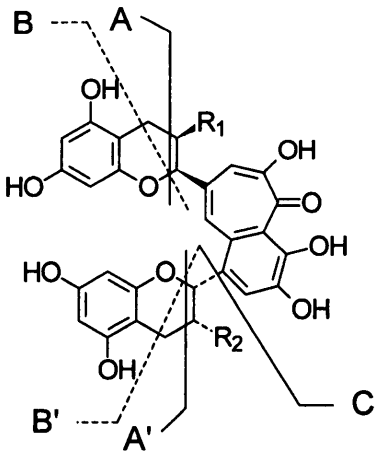


Figure 3. HPLC and reconstructed mass chromatograms of theaflavin mixtures. A solvent system of acetonitrile-2% acetic acid at a flow rate of 1.7 ml/min was used for LC/MS analysis. Peak number designation: same as in Figure 1.

of purchased theaflavin mixture and the theaflavin extract from black tea using a 10 cm x 4.6 mm i.d. SilicaROD RP-18 HPLC column coupled with isocratic elution with a mobile phase of either acetonitrile-1% citric acid or acetonitrile-2% acetic acid at a flow rate of 2.5 ml/min. Peaks 1, 2, 3, and 4 are designated to theaflavin, theaflavin-3-monogallate, theaflavin-3'-monogallate, and



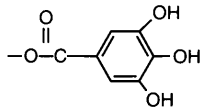
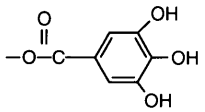
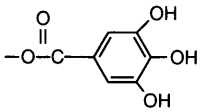
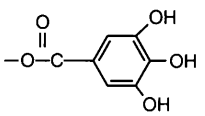
peak no.	compound	*m/z*
1	theaflavin R_1=OH, R_2=OH	565 $[M+H]^+$ 427 $[M+H-A \text{ or } A']^+$
2	theaflavin-3-monogallate R_1=  R_2=OH	717 $[M+H]^+$ 699 $[M+H - H_2O]^+$ 579 $[M+H - A \text{ or } A']^+$ 547 $[M+H - \text{gallate} - H]^+$ 409 $[M+H - \text{gallate} - H - A \text{ or } A']^+$
3	theaflavin-3'-monogallate R_1=OH R_2= 	717 $[M+H]^+$ 579 $[M+H - A \text{ or } A']^+$ 549 $[M+H - \text{gallate} + H]^+$ 547 $[M+H - \text{gallate} - H]^+$ 411 $[M+H - \text{gallate} + H - A \text{ or } A']^+$ 409 $[M+H - \text{gallate} - H - A \text{ or } A']^+$ 333 $[M+H - C - H]^+$
4	theaflavin-3,3'-digallate R_1=  R_2= 	869 $[M+H]^+$ 731 $[M+H - A \text{ or } A']^+$ 701 $[M+H - \text{gallate} + H]^+$ 699 $[M+H - \text{gallate} - H]^+$ 575 $[M+H - \text{gallate} - H - B \text{ or } B']^+$ 563 $[M+H - \text{gallate} + H - A \text{ or } A']^+$ 563 $[M+H - \text{gallate} - H - A \text{ or } A']^+$ 333 M+H - C - H]$^+$

Figure 4. Proposed fragmentation of the theaflavins by Atmosphere Pressure Chemical Ionization-Mass Spectrometry in the positive ion mode.

theaflavin-3,3'-digallate, respectively, based on reported data in literatures [4-6] and the mass spectral data as shown in Figure 2. The reversed-phase column coupled with gradient elution with solvent systems of acetonitrile-citric acid and acetonitrile-acetic acid has been commonly used for theaflavins analysis [3-6]. However, some have found HPLC methods to be time-consuming and unable to effectively separate theaflavin-3'-monogallate from theaflavin-3,3'-digallate. In this study, we demonstrated a rapid HPLC method with base-line separation of every individual theaflavin in 10 min by isocratic elution with conventional solvent systems.

II. *HPLC/MS Analysis*

When using HPLC/APCI-MS with heated-nebulizer interface, a flow rate higher than 2.0 ml/min and non-volatile solvent systems should be avoided. Therefore, we selected a mobile phase of acetonitile-2.0% acetic acid (21:79, v/v) at a flow rate of 1.7 ml/min for HPLC/MS analysis. Figure 3 shows the HPLC and reconstructed mass chromatograms in positive ion mode. The quasi-molecular ions $[M+H]^+$ of 565 (peak 1), 717 (peaks 2 and 3), and 869 (peak 4), corresponding to peaks 1, 2, 3, and 4 in the UV280. HPLC and Total Ion Count (TIC) chromatograms, were observed when a sample cone voltage of 10 V was applied. This $[M+H]^+$ was formed via a proton $[H]^+$ transfer from a protonated solvent molecule, which was obtained when the polar solvent used as a mobile phase was protonated at atmospheric pressure.

In order to obtain more fragment ions for more structural information about the analytes, a higher sample cone voltage of 40V was introduced. The mass spectra of these four major theaflavins are demonstrated in Figure 2. Quasi-molecular ions of these four theaflavins still remained at a significant percentage even when a higher cone voltage (40V) was applied. The proposed fragmentations of these four theaflavins are presented in Figure 4. Ring cleavage of A or A' (-138) could occur on these four protonated theaflavins. The loss of gallate fragment followed by obtaining or losing a proton (-168 or 170) occurred on protonated theaflavin-3-monogallate, theaflavin-3'-monogallate, and theaflavin-3,3'-digallate. The loss of gallate fragment followed by the cleavage of A or A' is also a common fragmentation pathway for the protonated gallate-containing theaflavins. The most abundant ion in the mass spectra of theaflavin-3,3'-digallate (peak 4) is m/z 575 as shown in Figure 2. This ion is obtained by the loss of gallate fragment and a proton followed by the cleavage of B or B' as proposed in Figure 4. Ion m/z 333 corresponds to a fragment ion generated from protonated theaflavin-3'-monogallate or theaflavin-3,3'-digallate by the cleavage of fragment C followed by the loss of a proton. This m/z 333 is, therefore, considered as a characteristic fragment ion from protonated theaflavins containing the gallate group at R_2 position. This ion can also be used to distinguish theaflavin-3'-monogallate from theaflavin-3-monogallate. The mass spectral data of theaflavin-3-monogallate (peak 2) and theaflavin-3'-monogallate (peak 3) in Figures 2 and 4 show that m/z 411 and 549 are also the characteristic ions from protonated theaflavin-3'-monogallate. This result implies that, in comparison with protonated theaflavin-3-monogallate, protonated theaflavin-3'-monogallate is much easier to get a proton after losing gallate fragment.

In this study, we demonstrate that theaflavins can be efficiently separated by a SilicaROD RP-18 column and easily analyzed by HPLC/APCI-MS without standards.

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以高效液相層析質譜儀分析紅茶中的茶黃質

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摘　要

本研究乃以10 cm X 4.6 mm i.d. SilicaROD RP-18高效液相層析(HPLC)分離管柱，配合乙腈-2%醋酸或乙腈-1%檸檬酸之流動相系統作等梯度沖提，可有效並快速分離紅茶中的茶黃質。其分析時間可於10分鐘內完成。茶黃質的鑑定工作乃以HPLC配合大氣壓化學離子化-質譜儀(APCI-MS)進行。利用正離子源作離子化，可於質譜圖上觀察到茶黃質的類分子離子(quasi-molecular ions, MH^+)峰及一些特殊的斷裂離子峰。本報告並提出茶黃質質譜斷裂離子峰之可能形成途徑。

關鍵詞：茶黃質，大氣壓化學離子化-質譜儀(APCI-MS)，液相層析質譜儀(LC/MS)。