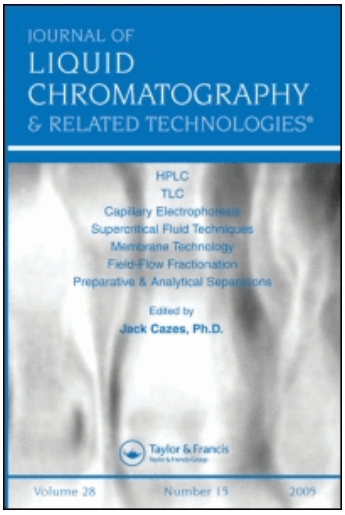

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 23 April 2002

To cite this Article Robb, Christina. S. , Geldart, Susan E. , Seelenbinder, John A. and Brown, Phyllis R.(2002) 'ANALYSIS OF GREEN TEA CONSTITUENTS BY HPLC-FTIR', Journal of Liquid Chromatography & Related Technologies, 25: 5, 787 — 801
To link to this Article: DOI: 10.1081/JLC-120003036
URL: http://dx.doi.org/10.1081/JLC-120003036

J. LIQ. CHROM. & REL. TECHNOL., 25(5), 787–801 (2002)

ANALYSIS OF GREEN TEA CONSTITUENTS BY HPLC-FTIR

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ABSTRACT

HPLC-FTIR was investigated for the qualitative determination of catechins and methyl xanthines present in green tea extracts. A reversed phase separation of the green tea components was performed on a C-18 column equilibrated at 30°C using an isocratic mobile phase of acetonitrile: 0.1% formic acid (15:85), prior to introduction to the deposition interface linked to the FTIR detector. The solvent was evaporated at 130°C and spectra were collected every six seconds during the run. Six catechins, (+)-catechin, gallocatechin, (−)-epicatechin, (−)-epigallocatechin and (−)-epigallocatechin gallate, and (−)-epicatechin gallate, as well as two methyl xanthines, caffeine and theobromine, were separated and positively identified in a sample of Chinese green tea. The catechins and methyl xanthines were matched to the respective standards by both retention time and the measured infrared spectrum.

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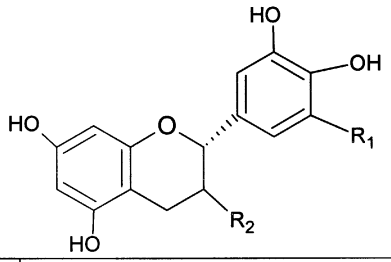
INTRODUCTION

Green tea is an increasingly popular beverage with several associated health benefits. Catechins (Table 1) make up about 30% of the chemical composition of green tea.(1) Catechins are effective antioxidants (2–5), that have been identified as preventative in the formation (6) and development of cancer (7–10) and cardiovascular disease (11). Anti-microbial effects of catechins have also been reported.(12) Green tea also contains about 3–6% of caffeine, a common stimulant.(1)

Traditionally, reversed phase HPLC has been used for tea analysis.(13) Catechin stability is pH dependent. The stability decreases as the pH increases (14); therefore, acidic mobile phases, with methanol or acetonitrile modifiers, are commonly used.(13) In addition, several HPLC methods developed for catechins have been optimized for the presence of caffeine.

Many HPLC detection methods have been applied to the identification of catechins. Single wavelength UV and photodiode array (15–33), fluorescence (27,29,32) electrochemical (25,29,33), chemiluminescence (34), and mass spectrometry detection (35–38) have all been employed. FTIR detection is suited to the detection of catechin compounds due to their high infrared activity. Furthermore, differences in the infrared spectra of catechins from different classes can be correlated with small structural changes between those classes.

Table 1. Catechin Structures



Catechin Compound	Chemical Name	R_1	R_2
Catechin	[2R,3S]-2[-3,4-Dihydroxyphenyl]-3,4-dihydro-1[2H]-benzopyran-3,5,7-triol	H	OH
Gallocatechin	[2R,3R]-2-[3,4,5-Triihydroxyphenyl]-3,4-dihydro-1[2H]-benzopyran-3,5,7-triol	OH	OH
Catechin Gallate	[2S,3R]-2-[3,4-Dihydroxyphenyl]-3,4-Dihydro-1-[2H]-Benzopyran-3,5,7-triol-3[3,4,5-trihydroxybenzoate]	H	1,2,3 trihydroxy benzoate

Two distinct designs for HPLC-FTIR interfaces have been developed: flow cells and solvent elimination systems.(39) Flow cell systems acquire spectra of the eluent in the solvent matrix through IR transparent, nonhydroscopic windows. The spectrum of the solvent is then subtracted from the sample spectrum, with the result being the spectrum of the analyte of interest. Solvent elimination systems nebulize the HPLC eluent, and remove the solvent before depositing the dried solutes onto an infrared transparent surface. Due to the strong infrared absorption of water and other solvents, solvent elimination systems are generally more sensitive than flow cell arrangements. The deposition of the analytes onto a surface also allows further characterization of the analyte to be performed, if necessary.

EXPERIMENTAL

Chemicals and Reagents

Standards of (+)-catechin, (−)-epicatechin, epicatechin gallate, epigallocatechin, gallocatechin, and epigallocatechin gallate were all obtained from Sigma Aldrich (Milwaukee, WI). Caffeine, theobromine, acetonitrile, and HPLC grade water were obtained from Fisher Scientific (Suwanee, GA). Liquid nitrogen and helium gas were obtained from Medical-Technical Gases Inc. (Medford, MA). Loose green tea was purchased in China.

HPLC Instrumentation and Methodology

The HPLC pump was from Thermo Separations Constametric 4100 (Austin, TX) and was used in conjunction with a LDC Analytical Membrane Degasser (Austin, TX). An Eppendorf CH-30 column heater (Brinkmann Instruments, Westbury, NY) was used to maintain column temperature. A Zorbax SB-C-18 (15 cm × 3 mm) column (Hewlett-Packard, Palo Alto, CA) was used for the separation.

The HPLC method used for the analysis of the tea extracts was 85:15, 0.1% formic acid: acetonitrile at a column temperature of 50°C using the Zorbax column. The flow-rate was 0.2 mL/min.

FTIR Detection

In this study, the Infrared Chromatograph (IRC) (Bourne Scientific, Acton, MA) was used. A detailed description of the IRC is provided elsewhere.(40) The

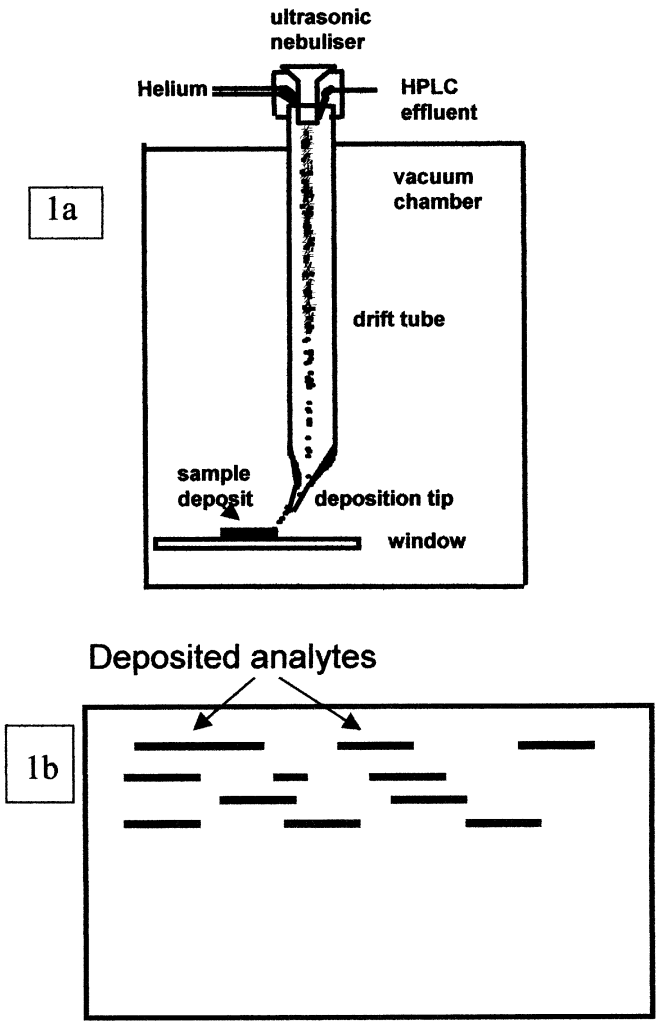


Figure 1. a) Schematic of the drift tube, b) Schematic of the zinc selenide plate.

IRC nebulized the HPLC effluent with an ultrasonic nebulizer (Figure 1A). The effluent from the HPLC was split so that 0.08 mL/min of effluent was delivered to the ultrasonic nebulizer. The mist traversed the heated evaporation tube (drift tube) under a helium flow of about 1 L/minute. The drift tube was held at a constant temperature of 130°C to remove the solvent, resulting in a dried analyte stream. The analytes were deposited onto a zinc selenide (ZnSe) plate

(2×1.5 inches) contained within the vacuum chamber. The ZnSe plate is part of a continuously moving stage, such that analytes are deposited in linear tracks. The stage moved at a rate of 2 mm/minute; therefore each part of the chromatogram occupied a different position on the plate. The focused IR beam passed through the sample track approximately 2 mm from the deposition tip. IR radiation was measured with a liquid nitrogen cooled MCT detector. Spectra were collected at a rate of 10 spectra per minute; each spectrum contained 24 co-added scans. The spectra were displayed in real time upon the computer screen as the run proceeded. At a scan rate of 10 scans per minute, the plate collected 15 hours of chromatographic data. When the ZnSe plate was full, it was removed and wiped clean with methanol.

Ultra-Violet Detection

A 3200 UV spectrometer SpectroMonitor monitored the excess effluent from the chromatograph. A SP4270 integrator from Spectra-Physics integrator recorded the UV data at a wavelength of 270 nm. Both instruments were from Thermo Separations (Austin, TX).

Standard and Sample Preparation

Stock standard solutions of the 6 catechins, theobromine, and caffeine were prepared at a concentration of 1 mg/mL each, in separate amber vials. Tea samples were prepared by the addition of 2 grams of loose tea to 100 mL of boiling water for 5 minutes. The tea was then allowed to cool before filtration and analysis. All standards and samples were filtered through 0.45-μm filter paper (Gelman Inc, Medford, MA) before analysis.

RESULTS AND DISCUSSION

HPLC Green Tea Separations

The reproducibility of the HPLC separations was under 5% RSD. The retention order of the green tea constituents was found to be gallocatechin, theobromine, epigallocatechin, (+)-catechin, epicatechin, caffeine, epigallocatechingallate, and finally epicatechingallate. This retention order is consistent with prior studies, as is the separation time of 25 minutes (13). The retention times of the standards matched those of the constituents in tea.

Deposition Characteristics

The heart of the Bourne IRC HPLC-FTIR is the drift tube, which is the deposition interface, depositing the analytes onto the ZnSe plate. Optimal catechin deposits were made when the drift tube had been washed with formic acid prior to the catechin analysis being performed the same day. This procedure minimized the adhesion of the highly hydroxylated catechins to the inner surface of the drift tube.

Infrared Spectral Characteristics of Catechin Standards

Figure 2 shows the full spectrum of the (+)-catechin standard obtained from HPLC-FTIR. All the spectra of the catechins investigated shared certain spectral similarities. A broad band due to the OH stretch is observed near 3350 cm^{-1}. A band due to the aromatic ring quadrant stretch is observed at 1618 cm^{-1}, and one due to the aromatic semicircle stretch at 1520 cm^{-1}. At 1280 cm^{-1}, a band due to the OH deformation of the aromatic alcohol is observed, and at 1190 cm^{-1}, a band corresponding to the CO stretch of an aromatic alcohol is observed. Near 1090 cm^{-1}, another band due to an aromatic ring stretch is observed. An aliphatic secondary alcohol, CO stretch, is observed near 1015 cm^{-1}. The bands due to the aromatic OH wags are observed between

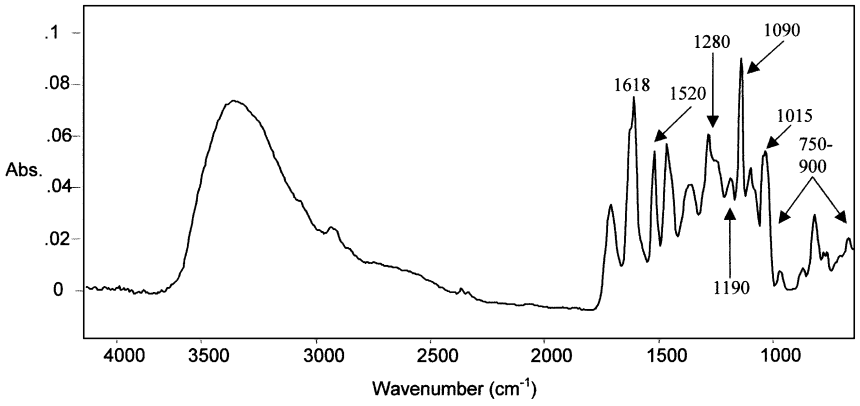


Figure 2. Full FTIR spectra of (+)-catechin obtained from the HPLC-FTIR using the Zorbax C18 column with 85:15% 0.1% formic acid: acetonitrile, 0.2 mL/min, column temperature 50°C.

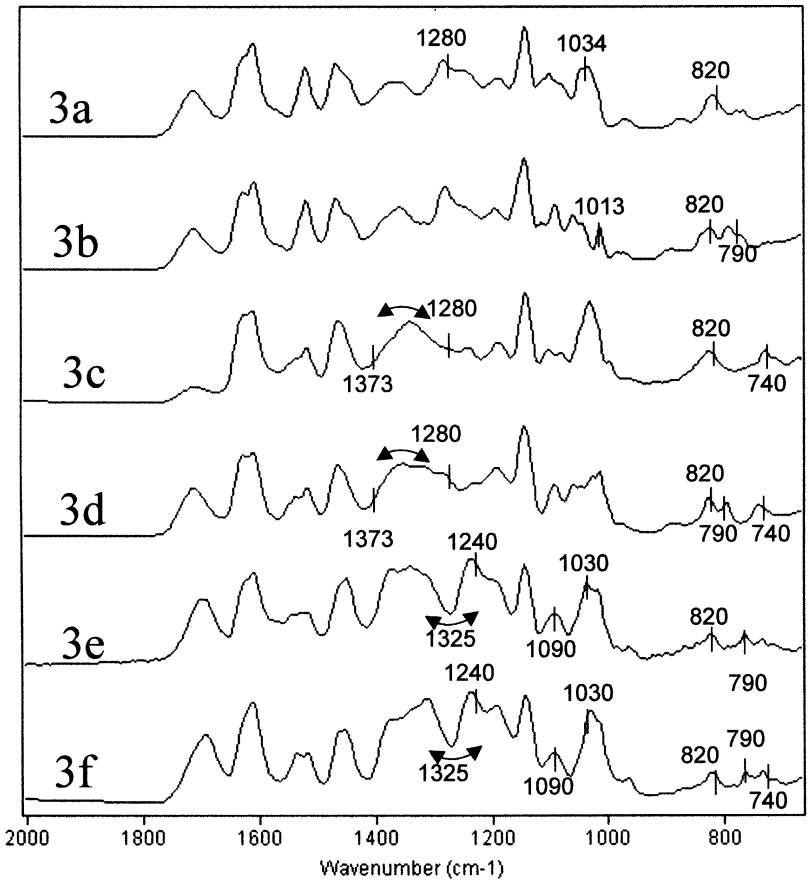


Figure 3. FTIR spectra of catechin standards obtained from the HPLC-FTIR. a) (+)-catechin, b) (−)-epicatechin, c) gallocatechin, d) (−)-epigallocatechin, e) (−)-epicatechingallate, f) (−)-epigallocatechingallate. Same analysis conditions as Figure 2.

900–750 cm^{-1}; the frequency of the bands due to the OH wags depend on the substitution of the aromatic rings.

Figure 3 shows the infrared spectra of (+)-catechin, (−)-epicatechin, (−)-gallocatechin, epigallocatechin, (−)-epicatechingallate, and (−)-epigallocatechingallate, listed as 3a–3f, respectively. All the infrared spectra were obtained from deposition of catechin standards on the HPLC-FTIR.

(+)-Catechin (Figure 3a) and (−)-epicatechin (Figure 3b) are diasteromers. In epicatechin, the OH is *syn* to the adjacent aliphatic ring, whereas in (+)-

catechin, the OH is *anti* to the ring. Two spectral differences result from this subtle structural difference. The aromatic CH wag of (+)-catechin is observed at 820 cm^{-1}, whereas the spectrum of epicatechin shows two bands at 820 cm^{-1} and 790 cm^{-1}. In addition, the aliphatic CO stretch in the *syn* conformation is observed at 1013 cm^{-1}, whereas the stretch in the anticonformation is observed at a higher energy of 1034 cm^{-1}.

(+)-Catechin (Figure 3a) and gallocatechin (Figure 3c) differ by the presence of a hydroxyl group on the R_1 position. The following spectral changes in the aromatic OH deformation and CO stretch are observed. First, the band due to OH deformation stretch is much sharper at 1280 cm^{-1} in the spectrum of (+)-catechin, whereas in the spectrum of gallocatechin it forms a broad band from 1373 to 1280 cm^{-1}. In addition, the gallocatechin also contains an additional band near 740 cm^{-1} due to the out of plane CH wag of the aromatic ring that is not observed in the (+)-catechin. The same differences are observed in the spectra of epicatechin (Figure 3b) and epigallocatechin (Figure 3d).

The spectra of (−)-epicatechin (Figure 3b) and (−)-epicatechingallate (Figure 3e) standards are also shown. The gallate is a trihydroxybenzoate of epicatechin. Both spectra contain the expected bands due to the aromatic CH wags at 820 and 790 cm^{-1} indicative of the epi form of the isomer. However, in the spectrum of epicatechingallate, there is a strong band at 1240 cm^{-1} and broad bands at 1090 cm^{-1} and 1030 cm^{-1} that correspond to the additional hydroxy groups in the gallate moiety.

The spectrum of EGCG (Figure 3f) has contributions from both the gallo and gallate groups. The bands at 1240, 1090, and 1030 cm^{-1} are indicative of the trihydroxybenzoate as seen in the epicatechingallate. The aromatic ring band at 740 cm^{-1} from the gallo moiety is also observed. Finally, there is a similar split profile of the band centered at approximately 1325 cm^{-1}, which is also observed in epicatechingallate.

Infrared Spectral Characteristics of Catechins in Tea Extracts

Figure 4a shows the peak chromatogram of a green tea extract analyzed by the IRC. This is the original chromatogram that is displayed while the run progresses, and is generated by plotting the absorbance of the highest absorbing band in each spectrum. It provides near universal detection of organic compounds, as it is independent of any particular band. However, selectivity for a particular compound or family of compounds can be observed by the creation of a band chromatogram. A band chromatogram is the chromatogram at a specific infrared frequency and is generated with the IRC software. Figure 4b shows a band chromatogram generated at 1519 cm^{-1} from the peak chromatogram of the green tea shown in Figure 4a. The band at

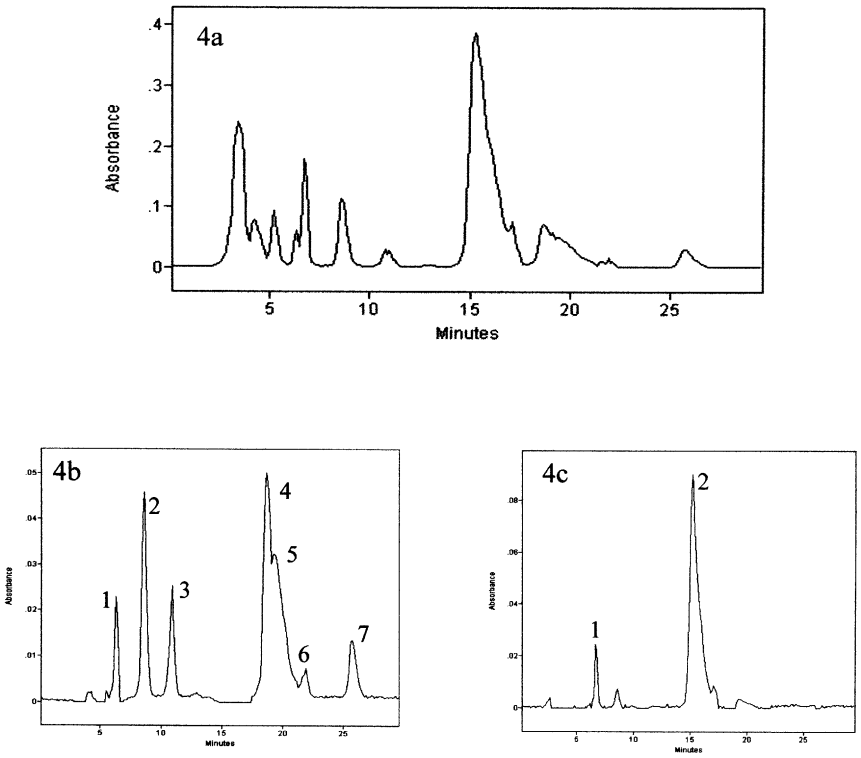


Figure 4. a) Peak chromatogram of green tea extract. b) Band chromatogram at 1519 cm^{-1} 1) Gallocatechin, 2) Epigallocatechin, 3) Catechin, 4) Epicatechin, 5) Epigallocatechingallate, 6) Epicatechingallate. c) Band chromatogram at 1565 cm^{-1}, 1) Theobromine, 2) Caffeine. Experimental conditions the same as Figure 2.

1519 cm^{-1} was selected, as it was only present in the catechins and not in the methyl xanthines, thus, the catechins are more clearly defined than in the original peak chromatogram.

Seven catechin peaks could be identified from the band chromatogram. Figure 5 shows the infrared spectra of components with the spectra of the corresponding standards. Figure 5a is the spectrum of the component that eluted at 6.3 minutes. The spectrum of gallocatechin is a clear positive match for this component (Figure 5b). Figure 5c is the spectrum of the component that eluted at 8.6 minutes which matches the standard spectrum of (−)-epigallocatechin (Figure 5d). Figure 5e is the spectrum of the component that eluted at 10.86 minutes. The component clearly matches the spectrum of (+)-catechin, which is

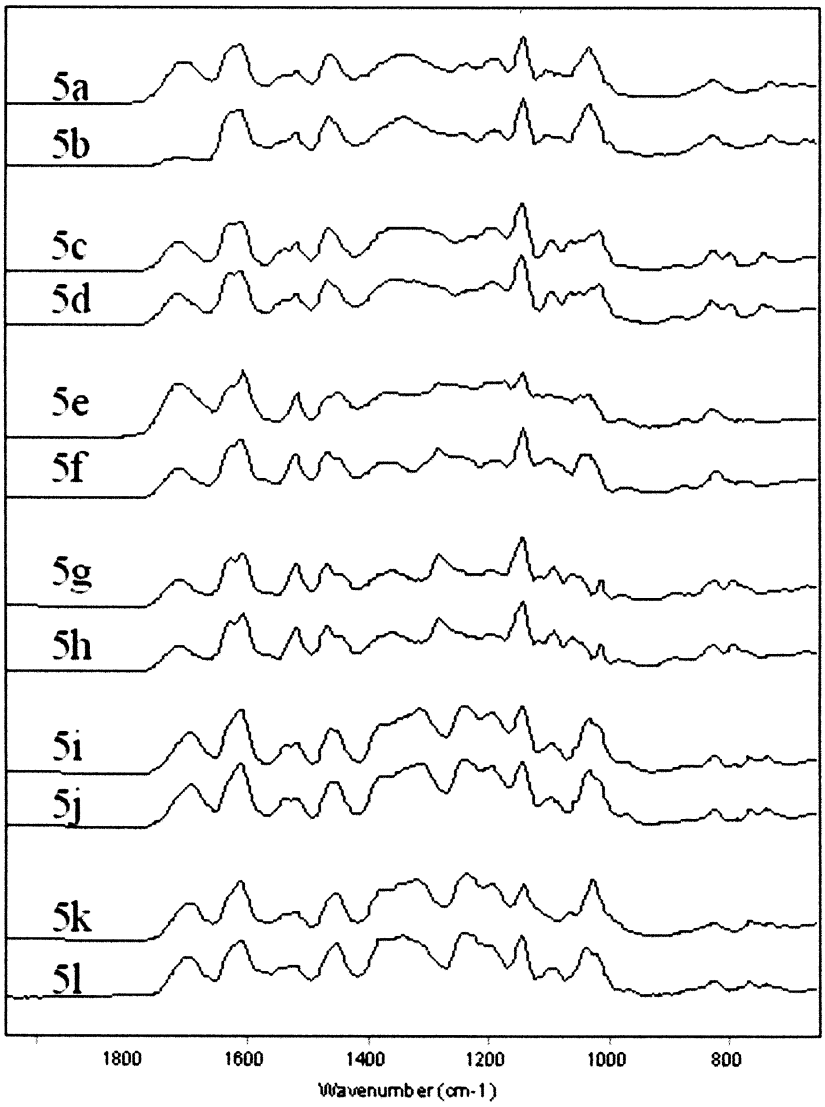


Figure 5. (a) Spectrum of component which eluted 6.3 minutes. (b) Spectrum of gallocatechin standard. (c) Spectrum of component which eluted at 8.6 minutes. (d) Spectrum of epigallocatechin standard. (e) Spectrum of component which eluted at 10.86 minutes. (f) Spectrum of (+)-catechin standard. (g) Spectrum of component, which eluted at 18.63 minutes. (h) Spectrum of epicatechin standard. (i) Spectrum of component, which eluted at 19.19 minutes. (j) Spectrum of epigallocatechingallate standard. (k) Spectrum of component, which eluted at 25 minutes. (l) Spectrum of epicatechingallate.

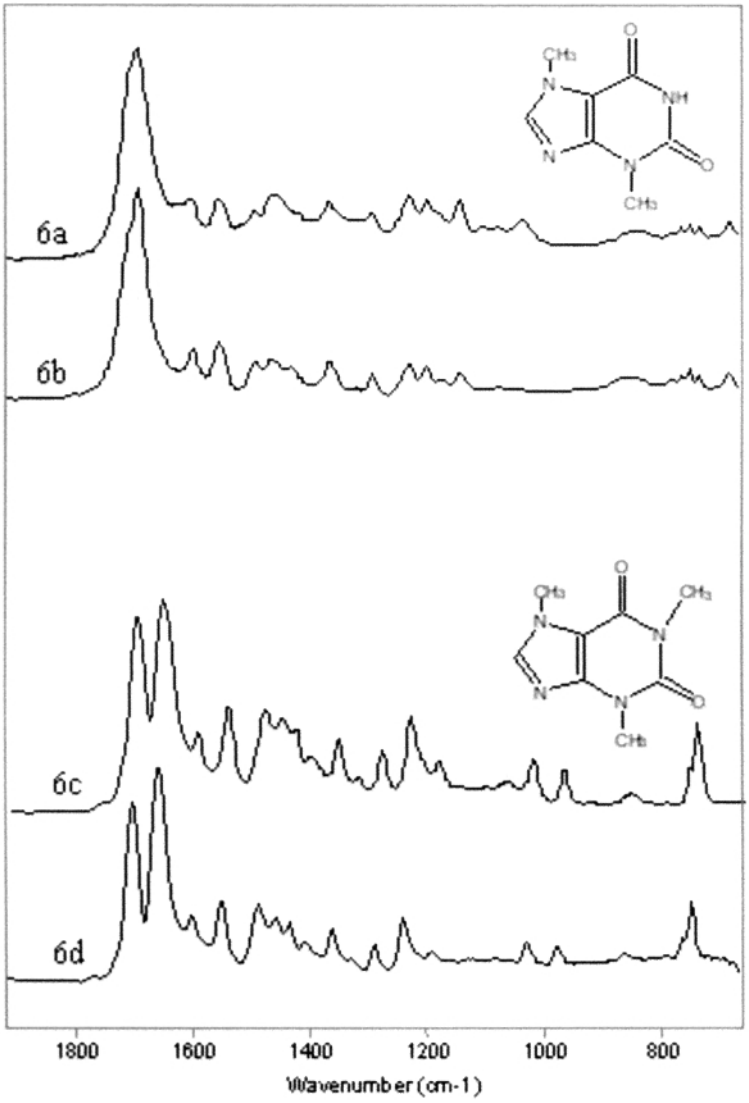


Figure 6. Spectra of standard methyl xanthines and matching components in green tea. a). Spectrum taken at 6.8 minutes. b). Standard spectrum of theobromine. c). Spectrum taken at 15.5 minutes. d). Standard spectrum of caffeine.

shown in Figure 5f. Figures 5g and 5h show the spectra of the component at 18.63 minutes and the (−)-epicatechin standard respectively. Figures 5i and 5j show the spectra of the component at 19.19 minutes and the (−)-epigallocatechingallate standard. In both cases, clear positive matches are evident. Figures 5k and 5l show the spectra of the component at 25.00 minutes and the (−)-epicatechingallate standard. The retention times of all the catechin constituents of the tea extracts were found to match those of the catechin standards.

Infrared Spectral Characteristics of Methyl Xanthines in Tea Extracts

The methyl xanthines, theobromine and caffeine, were also separated from the green tea extract. Figure 4c displays the band chromatogram plotted using the intensity of the band at 1565 cm^{-1}. Theobromine and caffeine eluted at 6.8 minutes and 15.25 minutes, respectively. Standards measured under the same conditions had identical retention times. The similar methyl xanthines can also be distinguished by their infrared spectra. Infrared spectra of theobromine and caffeine from both standards and the green tea extract are shown in Figure 6. Though their structures are similar, the IR spectra of the two compounds differ in the carbonyl stretch region. In the spectra of theobromine, a CO stretch due to the imide functionality is observed at 1690 cm^{-1}. In the infrared spectra of caffeine, however, two bands are observed in the carbonyl region. The band at 1700 cm^{-1} is due to the CO stretch of the disubstituted amide, while the band at 1656 cm^{-1} is due to the CO stretch of the urea. A clear positive match of the standard methyl xanthines to the components in the green tea extract is evident.

CONCLUSIONS

This study has shown that HPLC-FTIR can be used to qualitatively analyze and identify catechins and methyl xanthines in green tea extracts. Acid washing the drift tube was recognized as being essential to obtaining reproducible catechin depositions. With infrared detection, clear structural identification of the different catechins was made and structural differences between catechin classes were identified. (+)-Catechin, gallocatechins, and gallated catechins, were distinguished, and even subtle structural differences such as (+)-catechin and (−)-epicatechin, which only differ by the *syn*/*anti* orientation of a hydroxyl substitute, were clearly identified.

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Received October 1, 2001
Accepted November 1, 2001
Manuscript 5667