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Selection of column and gradient elution system for the separation of catechins in green tea using high-performance liquid chromatography

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Abstract

A study of a variety of stationary phases and elution conditions for the liquid chromatographic (LC) determination of six biologically active green tea catechins has resulted in the development of two well-defined, reproducible systems for such analyses which overcome limitations of previously described methods. Comparison of six reversed-phase columns indicates that deactivated stationary phases, which utilize ultrapure silica and maximize coverage of the silica support, provide significantly improved separation and chromatographic efficiencies for catechin analyses using LC, compared to conventional monomeric or polymeric C_{18} columns. Evaluation of elution conditions used for the separations reveals that the presence of acid in the mobile phase (0.05% trifluoroacetic acid) is essential for both the complete resolution of the catechins present in tea and the efficient chromatography of these compounds. The efficacy of one of the developed systems was demonstrated by the quantitative measurement of the six biologically active catechins in aqueous infusions of green tea (*Camellia sinensis*). Overall precision values for the analyses were within the range 0.3–1% (relative standard deviation).

Keywords: Stationary phases, LC; Green tea; Gradient elution; Catechins; Polyphenols

1. Introduction

The notion that specific dietary practices may provide our best protection against cancer and cardiovascular disease has received significant support from a number of studies within the last five years [1–3]. Most recently, the relationship between tea consumption and prevention of certain forms of human cancer has received a great deal of attention [4,5], although epidemiological studies concerning the effect of tea consumption on human cancer risk have been inconsonant [6]. Nevertheless, several recent laboratory studies have provided indisputable evidence supporting a role for tea and tea polyphenols (catechins) in the inhibition of cancer in animal models [6]. The six major tea catechins known to display biological activity are (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-gallocatechin gallate (GCG), (−)-epigallocatechin gallate (EGCG), and (−)-epicatechin gallate (ECG) (Fig. 1). These natural products have strong antioxidant activity [7,8], and have been shown to exhibit numerous potentially beneficial medicinal properties including inhibition of carcinogenesis [9,10], tumorigenesis [11,12] and mutagenesis [8,13,14], as well as the inhibition of tumour

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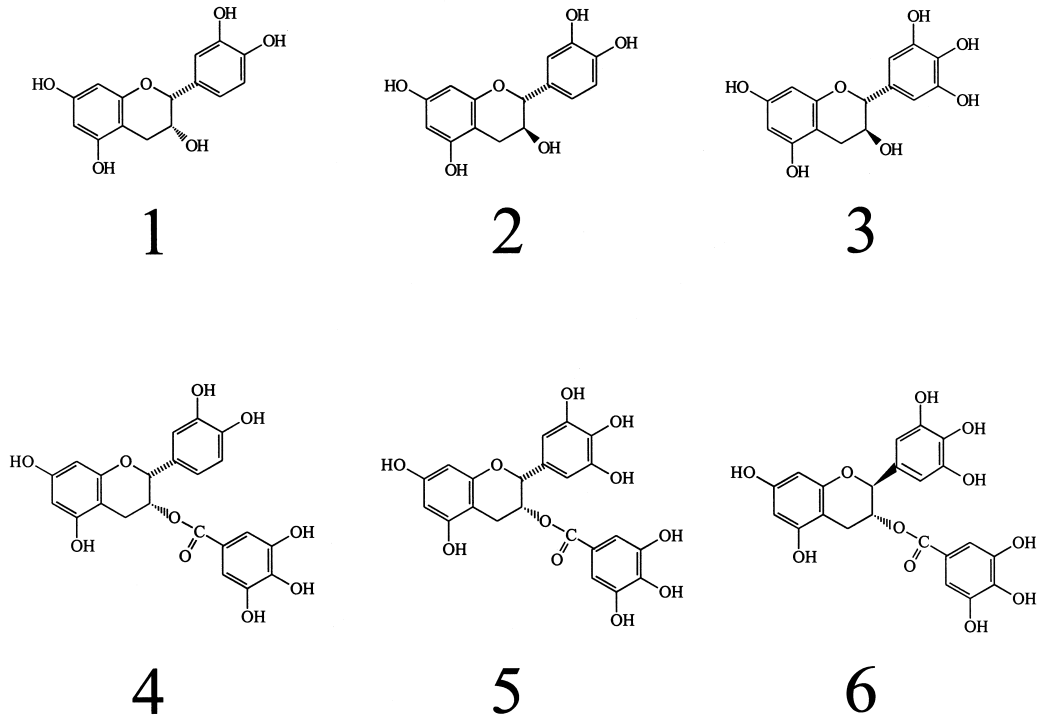

Fig. 1. Structures of six catechins from green tea. (1) (+)-catechin; (2) (−)-epicatechin; (3) (−)-epigallocatechin; (4) (−)-epicatechin gallate; (5) (−)-epigallocatechin gallate; (6) (−)-gallocatechin gallate.

growth and metastasis [15]. In addition, these bioflavonoids have antibacterial [16] and antiallergic [17] properties, and have been demonstrated to induce apoptosis in human lymphoid leukemia cells [18], inhibit platelet aggregation [19], and inhibit human immunodeficiency virus (HIV) reverse transcriptase [20].

The method of choice for the analysis of catechins in tea and biological matrices has traditionally been high-performance liquid chromatography (LC) [21–30], and to a lesser extent gas chromatography (GC) [31]. Recently, successful analysis of green tea catechins has also been reported using capillary electrophoresis [32,33].

Although the reversed-phase LC analyses referred to above all demonstrate separation and detection of green tea catechins with varying degrees of success, they all have notable limitations with regard to simple, accurate, and reliable qualitative and quantitative measurements. These include the use of complex mobile phases [23,26–28], failure to provide clean separation of the analytes from each other and/or from other compounds in the mixture (e.g., caffeine etc.) [23,26–28], requirement of pre-purification or extraction procedures prior to analysis [22,24,25,30], poor chromatographic efficiency [25,27–29], and with few exceptions [22,23,30], failure to address the resolution of the GCG *trans*-isomer from the EGCG *cis*-isomer and the EC *trans*-isomer from the C *cis*-isomer, reporting EGCG and EC as single pure components, when they may, in fact, contain low levels of their respective diastereomers. Further, the C_{18} solid supports and stationary phases used for nearly all of these analyses are not well-defined, and our attempts to reproduce the published chromatographic separations using conventional C_{18} columns have been largely ineffective.

We presently report on a systematic comparison of a variety of reversed-phase LC columns for the separation of catechins in green tea, using two straightforward gradient elution systems. The study was designed in order to better understand the

chromatographic behavior of green tea catechins, as well as to overcome the limitations of previously reported methods. Now in routine use, the analytical system which has been developed represents a simple and reliable method for the efficient separation and quantification of these interesting compounds.

2. Experimental[1]

2.1. Chemicals

(−)-Epigallocatechin (EGC), (+)-catechin (C), caffeine, (−)-epigallocatechin-3-gallate (EGCG), (−)-epicatechin (EC), (−)-gallocatechin gallate (GCG), (−)-epicatechin gallate (ECG), L-tryptophan, and trifluoroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade methanol (MeOH) and acetonitrile (ACN) were purchased from J.T. Baker (Baker–Mallinckrodt, Phillipsburg, NJ, USA). HPLC-grade water (18 mΩ), prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA, USA), was used to prepare all solutions.

2.2. Preparation of catechin standards

A standard aqueous solution containing 0.05 μg/μl of each of the six catechins and caffeine was prepared and used in all methods development and HPLC column comparison experiments.

2.3. Preparation of green tea samples

The green tea (Lung Ching) used in the present study was a gift from Yung Chi Wu and Sam A. Margolis of the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). The green tea samples were prepared using an aqueous extraction procedure which simulated actual brewing conditions for a cup of tea. A precisely known amount of green tea leaves was steeped at 80°C for 10 min in 20 ml of water containing a precisely known amount of the L-tryptophan internal standard. The samples were then filtered through a 0.45 μm nylon filter and analyzed directly by LC.

2.4. LC instrumentation, columns, and conditions

The LC system consisted of a liquid chromatograph connected to a variable wavelength UV detector. Data analysis was performed using a Dionex advanced computer interface with AI-450 data analysis software (Dionex Corp., Sunnyvale, CA, USA). The six reversed-phase LC columns studied represent commercially available C_{18} columns with the characteristics specified in Table 1.

Two gradient elution systems were developed utilizing different mobile phases for the separation of the seven major components of green tea. These two systems are summarized in Table 2. In all cases, the flow rate was 1.0 ml/min, and detection of analytes was accomplished with UV detection at 210 nm.

2.5. Construction of calibration curves for green tea catechins

To quantify each of the six catechins in green tea using the presently developed separation systems, calibration curves were constructed using primary standards of each analyte. For this standardization, three calibration mixtures were prepared for each catechin by mixing known amounts of primary standard and L-tryptophan internal standard solutions to achieve three different mass ratios in the mixtures. These solutions were then analyzed by LC with UV detection at 210 nm. The peak area ratios were calculated automatically using the data analysis software. The data were then subjected to a linear least squares analysis.

2.6. Measurement of catechin concentrations in green tea

Green tea samples were prepared containing a precisely known amount of internal standard as described in Section 2.3, prior to LC analysis. The peak area ratios (catechin/L-tryptophan) were then

[1]Certain commercial equipment, instruments or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the material or equipment identified are the best available for the purpose.

Table 1
Characteristics of the columns tested[a]

Column	Type	Description
A	Zorbax Eclipse XDB-C_{18} Rockland Technologies Inc./Dupont	Deactivated, endcapped monomeric C_{18}, ultrapure silica
B	Zorbax Rx-C_{18} Rockland Technologies Inc./Dupont	Deactivated, monomeric C_{18}, ultrapure silica
C	PAH Hypersil Keystone Scientific Inc.	Polymeric C_{18}
D	SMT OD-5-100 Separations Methods Technologies Inc.	Deactivated, horizontally polymerized mixed monomeric C_{18}–C_1
E	Phenomenex Ultracarb 5 ODS(20) Phenomenex Inc.	High carbon loaded monomeric C_{18}
F	Zorbax ODS-C_{18} Rockland Technologies Inc./Dupont	Monomeric C_{18}

[a]All columns are 4.6×250 mm I.D.×length, with 5 μm nominal particle size.

used in conjunction with the calibration curves to derive the amount of each catechin in green tea, expressed as mg/g green tea leaves.

3. Results and discussion

This study began with an attempt to reproduce several of the chromatographic separations found in the extant literature which describe the separation of the four most prominent catechins (EGC, EC, EGCG, and ECG), and to extend such analyses to include GCG and C. In general, it was found that the published separations were somewhat or entirely irreproducible, both with regard to complete resolution of the analytes and the quality of chromatography, especially when using conventional C_{18} columns (non-deactivated 5 μm ODS stationary phase). The reproducibility of these separations was also confounded by a lack of precise specification of stationary phases used in previous investigations. The lack of selectivity, broad peak widths, and severe tailing which we observed for all of the systems we tested, as well as our desire to include C and GCG in the analyses, necessitated a comparison of an assortment of reversed-phase C_{18} columns to better understand the chromatographic behaviour of green tea catechins, and the subsequent development of well-defined systems for the efficient chromatographic separation of these compounds.

3.1. Development of gradient elution systems and comparison of columns

Initial efforts to develop an isocratic elution system for the separation of catechins using a MeOH-based mobile phase were unsuccessful and resulted in poor resolution and chromatographic

Table 2
Gradient elution systems for separation of green tea catechins[a]

System	Buffer A	Buffer B	Gradient	
			Time (min)	%B
1	Water+0.05% TFA	Acetonitrile+0.05% TFA	0	12
			25	21
			30	25
			35	100
2	Water+0.05% TFA	60:40 MeOH–ACN+0.05% TFA	0	10
			5	15
			50	40

[a] All separations performed at room temperature.

efficiencies on the order of $N_{AVG} \cong 1000$. These efforts were subsequently shifted toward developing gradient elution systems which would overcome the chromatographic limitations described above, and provide baseline separation of all six catechins and caffeine. Two such systems were developed and are summarized in Table 2. Two notable points emerged during the development of these systems. (1) Complete separation of the catechins and chromatographic quality are column-dependent, preferring deactivated monomeric C_{18} columns to non-deactivated monomeric or non-deactivated polymeric C_{18} columns. (2) The presence of acid in the mobile phase is essential to both complete resolution of the catechins present in the mixture and efficient chromatography of these compounds, specifically, the elimination of peak tailing.

The first point is illustrated in Fig. 2 (elution system 1) and Fig. 3 (elution system 2). Columns A, B, and D (Table 1), which can be generally considered as deactivated columns, provide complete separation of all of the catechins as well as caffeine using system 1 (Fig. 2), whereas columns C, E, and F do not. The use of conventional monomeric C_{18} columns (Fig. 2E and F) results in a lack of complete separation of the seven components and significant peak tailing, probably caused by unfavorable interactions of the catechins with accessible acidic silanols. The peak shapes for all of the components of the mixture except caffeine are quite reasonable using a polymeric C_{18} column (Fig. 2C), but complete resolution of all seven analytes is not achieved, and there are interfering species coeluting with EGC (Fig. 2C, peak 1).

In general, columns A, B, and D are equivalent with regard to the chromatographic separations

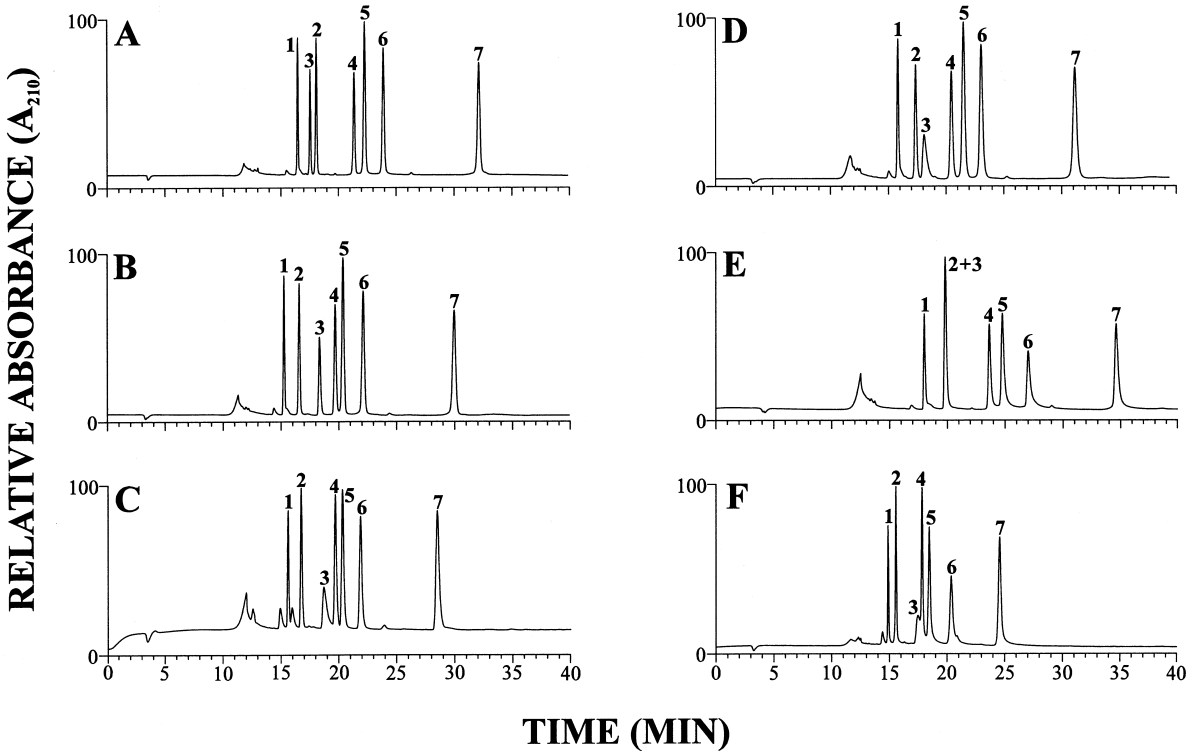

Fig. 2. Comparison of six reversed-phase HPLC columns for the chromatographic separation of six catechins and caffeine in a standard mixture using gradient elution system 1 (see Table 2). A description of each column (A–F) is given in Table 1. The concentration of each catechin in the standard mixture was 0.05 μg/μl. Detection was carried out with UV at A_{210}. Peak identification: (1) epigallocatechin (EGC); (2) (+)-catechin (C); (3) caffeine; (4) epicatechin (EC); (5) epigallocatechin gallate (EGCG); (6) gallocatechin gallate (GCG); (7) epicatechin gallate (ECG).

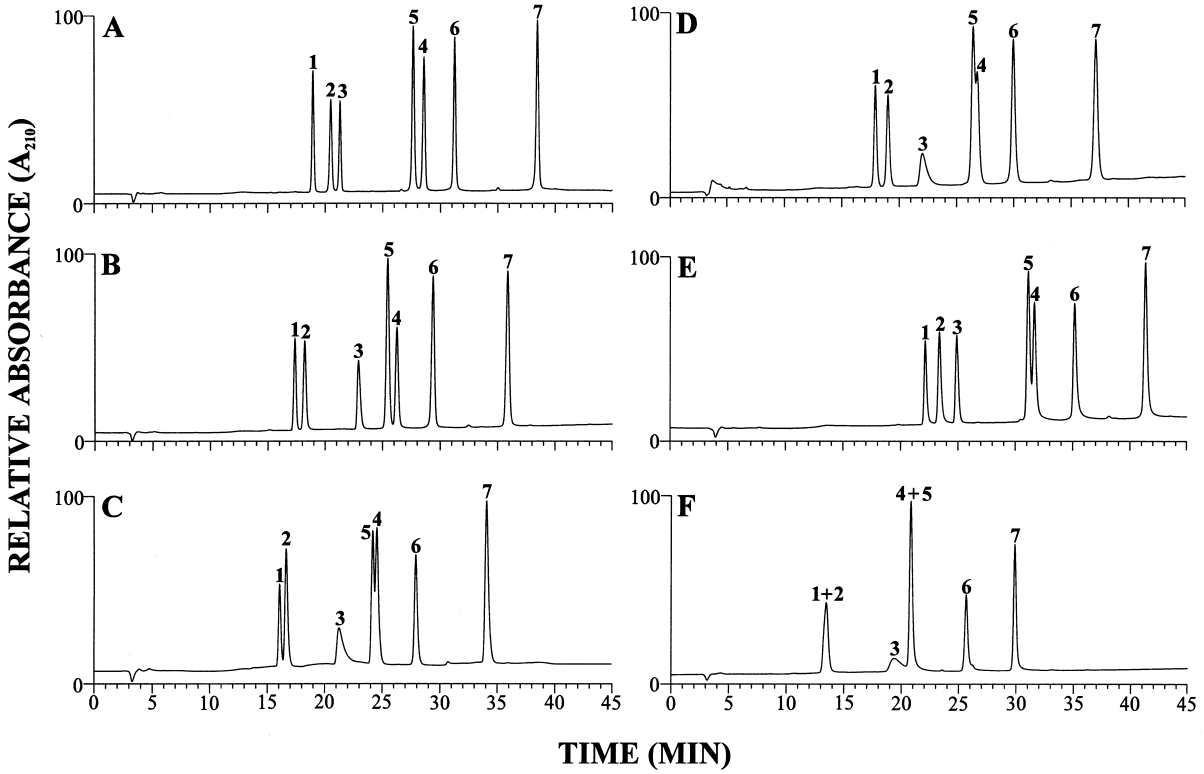

Fig. 3. Comparison of six reversed-phase HPLC columns for the chromatographic separation of six catechins and caffeine in a standard mixture using gradient elution system 2 (see Table 2). A description of each column (A–F) is given in Table 1. The concentration of each catechin in the standard mixture was 0.05 μg/μl. Detection was carried out with UV at A_{210}. Peak identification is the same as in Fig. 2.

utilizing system 1; however, the average bandwidth for column D (0.91 s) is significantly larger than that of columns A or B (0.47 s and 0.54 s, respectively). The elution of caffeine is particularly inefficient when using column D, indicating a difference in the mode of interaction of analytes with column D compared with columns A and B. It is clear from Fig. 2 that column A provides the best separation of all seven components and the best column efficiency. The reason columns A, B, and D provide improved chromatography for this class of compounds compared to conventional monomeric or polymeric C_{18} columns is presumably because they are designed to eliminate undesirable interactions between analytes and the silica surface, thereby providing enhanced selectivity and elimination of peak tailing. In the case of columns A and B, this is accomplished using an ultrapure and inert silica support [34]. Column A, which provides the highest quality chromatography of any of the columns tested, in addition to using ultrapure silica, further deactivates the C_{18} chains through endcapping and provides higher surface coverage than column B. For column D, deactivation is accomplished by essentially covering the entire silica surface using a single horizontally polymerized layer of mixed monomeric C_{18}–C_1 stationary phase [35].

Although a system for the separation of the seven major components of green tea using a conventional monomeric ODS column thermostatted at 40°C has been described [30], it has been our experience that no manipulation of the gradient or mobile phases for either system 1 or system 2 has resulted in complete separation of the seven components using columns E or F (non-deactivated monomeric ODS columns) at room temperature.

In developing gradient elution system 2, the volume fraction of MeOH in buffer B (a MeOH–

ACN mixture) was varied from 0 to 100% in 10% increments. The volume ratio 60:40 MeOH–ACN for buffer B was the only combination which resulted in complete separation of all seven analytes, and the presence of ACN in the buffer B mixture significantly improved chromatographic efficiency. A comparison of column utility using system 2 (Fig. 3) demonstrates that this elution system provides greater retention than system 1 and that only the deactivated columns A and B provide complete separation of all seven analytes. While the peak shape and chromatographic quality are remarkable for these columns (Fig. 3A and 3B), column D appears less well-suited for the complete separation of catechins using system 2. Even when minor manipulations were applied to the gradient and mobile phase for this system, complete separation could not be achieved.

The second major point that emerged from the development of well-defined LC systems for the separation of catechins is illustrated in Fig. 4, which shows the effect of the presence of acid in the mobile phase on the chromatography of these bioflavonoids. Fig. 4A and B show separations of the six catechins and caffeine using Column B and gradient elution system 1 in the presence and absence of 0.05% TFA, respectively. Fig. 4C and D depict a similar comparison using gradient elution system 2.

Comparison of these chromatograms indicates that the presence of acid is essential to the complete separation and elution of the seven analytes. This improvement in chromatography is also observed when 0.05% TFA is replaced with 0.5% acetic or formic acid, and therefore represents a pH effect, rather than an effect attributed specifically to TFA.

It should be noted that the chromatographic separations in the absence of acid depicted in Fig. 4B and D were performed on a brand new, equilibrated column which had not experienced prior acid treatment. Acid was then added to buffers A and B to generate the chromatograms illustrated in Fig. 4A and C. Interestingly, once the column had been equilibrated with acid containing buffers, subsequent separations using buffers in the absence of acid, even after extensive re-equilibration (>50 column volumes), were substantially improved, resulting in nearly complete separation of all seven compounds. However, tailing and irreversible retention of EGCG,

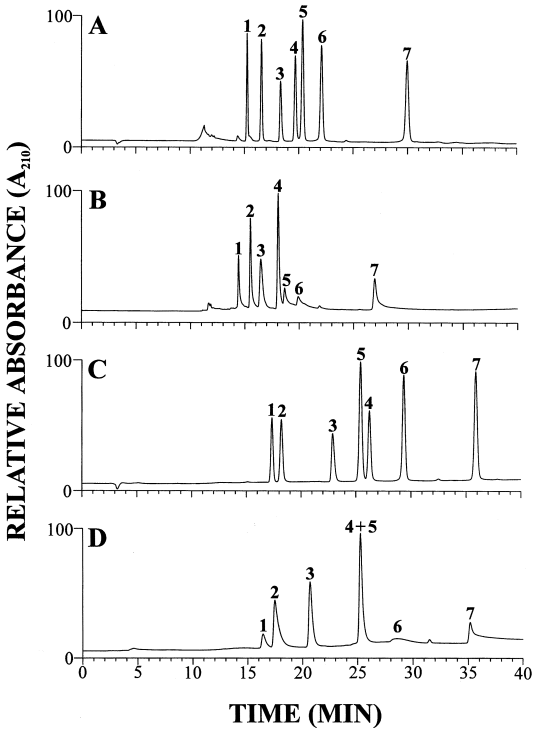

Fig. 4. The effect of acid on the separation of a standard catechin mixture using the deactivated monomeric C_{18} column B. (A) Elution system 1 using a mobile phase containing 0.05% TFA; (B) elution system 1 using a mobile phase without TFA; (C) elution system 2 using a mobile phase containing 0.05% TFA; (D) elution system 2 using a mobile phase without TFA. Peak identification is the same as in Fig. 2.

GCG, and ECG were still observed (Fig. 5). Similar chromatographic behaviour in the presence and absence of acid was observed using column A. This behaviour indicates that the presence of acid in the mobile phase has two distinct effects on catechin separations when using the deactivated monomeric C_{18} columns. First, the stationary phase is irreversibly altered by treatment with acid, resulting in increased chromatographic efficiency and performance for catechin separations. Second, the presence of acid in the mobile phase serves to help eliminate interactions of the bioflavonoids, especially EGCG, GCG, and ECG, with the solid support, further enhancing the overall quality of the separation. Finally, this laboratory has found that the use of acid-containing mobile phases in conjunction with deactivated monomeric C_{18} columns significantly

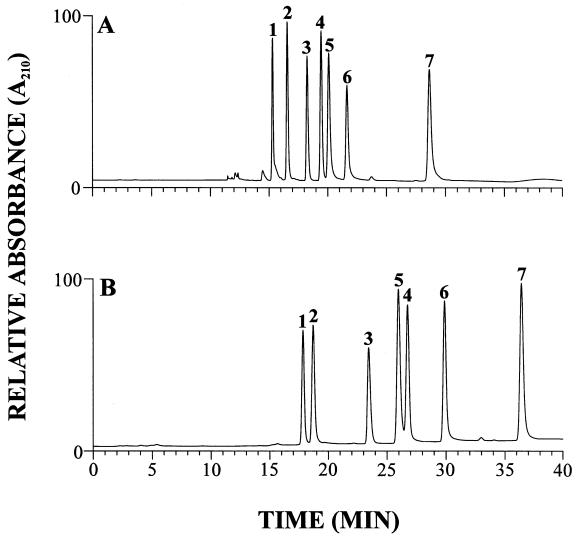

Fig. 5. Irreversible effect of acid treatment on the deactivated monomeric C_{18} column B. Column B, pre-treated with buffers containing 0.05% TFA, was equilibrated extensively with non-acid-containing buffers prior to analysis using (A) elution system 1, no TFA; (B) elution system 2, no TFA.

improves the chromatographic separations observed for other classes of bioflavonoid natural products as well [36].

3.2. Quantitative measurement of six catechins in green tea

Calibration curves using the internal standard L-tryptophan were constructed for each of the six biologically active catechins and used to quantify each in Lung Ching green tea using column A and elution system 2 (Table 3). Correlation coefficients for the curves were all >0.995. A typical chromatogram illustrating the separation of the catechins, caffeine, and L-tryptophan in a green tea infusion is illustrated in Fig. 6. Three replicate measurements were made for each catechin in one aqueous tea infusion. Carry-over in the LC system during sequential analyses of catechin samples was evaluated by running a blank LC experiment following the analysis of the standard catechin mixture. No memory effect was observed during the blank runs. Mean values and relative standard deviations were calculated for each catechin and are tabulated in Table 3. The calculations of catechin content in Lung Ching tea have precisions ranging from 0.3 to 1%. The analytical levels of each catechin shown in Table 3 were independently determined using micellar electrokinetic capillary chromatography (MECC) [33] with good agreement. To our knowledge, these are the first reported levels of catechins in green tea infusions which simulate an actual cup of tea. As such, comparison with previously LC-determined values calculated for chemical extractions of total catechins or aqueous extractions of bulk green tea leaves is unwarranted.

Table 3
Quantitation of the six catechins in green tea

Catechin	Concentration [mg catechin/g tea leaves]
EGC	15.4 (0.16)[a]
(+)-C	0.600 (0.004)
EGCG	21.7 (0.14)
EC	3.76 (0.03)
GCG	0.621 (0.002)
ECG	3.15 (0.02)

[a] Standard deviations (n=3) are given in parentheses.

4. Conclusions

The goal of the present work was to compare a variety of reversed-phase C_{18} HPLC columns to understand the chromatographic preferences of green tea catechins and to develop a well-defined and reliable system for their efficient separation and quantification. This report describes two such systems which take into account the chromatographic dependence of catechins on the use of deactivated monomeric C_{18} LC columns and gradient elution systems utilizing acid-containing buffers.

The present systems have several advantages over previously described methods including improved chromatographic efficiency, complete separation of all analytes including structural isomers, and the ability to analyze green tea infusions directly. Finally, the optimized systems developed use well-defined, commercially available LC columns, and uncomplex mobile phases, making the methods easily accessible, and adaptable to techniques such as directly combined liquid chromatography–mass spectrometry [37], which will provide increased

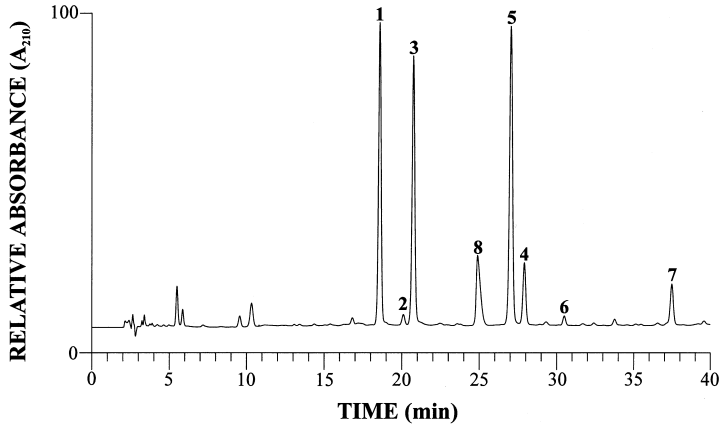

Fig. 6. Separation of catechins in an aqueous green tea infusion using a Zorbax Eclipse XDB-C_{18} column in conjunction with elution system 2 (Table 2). Peak identification is the same as in Fig. 2 except (8) L-tryptophan.

selectivity and sensitivity for the analysis of catechins in complex biological matrices.

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