Chapter 2

Development of a MEKC method for analyzing tea compounds

1.1 Introduction

HPLC is the most popular technique used to detect and quantify natural compounds that occur in green and black tea. Several HPLC methods exist for the analyses of different groups of tea compounds. The type of samples, extraction methods of catechins and the modes of detection vary between methods. Of importance is the analysis time required for HPLC analytical methods. The equilibration required between runs is generally 10 min long. With column equilibration all methods have an analysis time of 30 minutes or longer. The long duration of the methods limits the amount of samples that can be analyzed. A HPLC method was developed to analyze eight catechins as well as caffeine from green tea extracts (Goto, 1996). The analysis time was 20 minutes excluding column equilibration. A HPLC method was developed to analyze the five major catechins in green, oolong and black tea liquors (Khokhar, 1997). Eight catechins were analyzed in human plasma and saliva with a HPLC method developed by (Tsuchiya, 1997), (Tsuchiya, 1998). They made use of complex formation between diphenylborate and the vicinal hydroxyl groups of the catechins to achieve organic phase extraction and concentration of the polyphenols. This method significantly improved the LOD for catechins in samples. Without column equilibration the analysis time is 18 minutes. Five catechins could be detected in human plasma with a HPLC method developed by (Maiani, 1997). Sample treatment entailed HCl-methanol hydrolysis followed with ethyl acetate extraction. The method failed to separate caffeine from background analytes. Analysis of caffeine may be important in some bio-

medical applications, since both tea catechins and caffeine are substrates and regulators of P450 iso-enzymes (Ayalogu, 1995). The analysis time without column equilibration is 20 minutes. A HPLC method using chemiluminescence detection was developed to determine only EGCg in rat and human plasma (Nakagawa, 1997(a)). It is the most sensitive method to date, detecting EGCg in picomole quantities. All the methods above used reverse phase columns and gradients of increasing organic solvent concentrations to shorten the retention times of the more hydrophobic catechins. All the methods used UV detection except for the use of chemiluminescence (Nakagawa, 1997), electrochemical detection (Lee, 1995) and spectrofluorometry (Tsuchiya, 1998). In a study where fluorescence detection and normal UV detection were compared it was found that fluorescence improved the LOD more than 10 times without losing any specificity (Arts, 1998). Catechin concentrations could be detected in the ng/l range. Only one method exists for the determination of the total and enantiomeric (D/L forms) of theanine (Ekborg-Ott, 1997). The separation of D-L theanine was achieved with a γ-CD column used in the polar organic mode.

In both RP-HPLC and MEKC separation is achieved by the selective distribution of analytes between a long aliphatic carbon chain stationary/pseudo-stationary phase and a mobile aqueous/organic phase. Both techniques have the ability to separate neutral analytes. RP-HPLC does not have the ability to separate the charged molecules (anions, cations and zwitterions) since all of them elute on the void. We proposed that these charged molecules could be separated from each other with MEKC based on their different charge/mass ratios.

When we set out to develop a MEKC method to separate the different catechins, no other CE method separating tea compounds was available. During the course of the investigation several publications appeared where different tea components were analyzed with CE methods. At that time only several CE methods existed for the analysis of different flavonoids and flavonoid-O-glycosides with isotachophoresis (ITP) (Seitz, 1991), capillary zone electrophoresis (CZE) or micellar electrokinetic chromatography (MEKC). In all the publications the authors focused on the separation of a group of polyphenols and not a mixture of analytes with vastly different properties as found in tea extracts.

Closer scrutiny of the methods indicated four distinct groups of conditions used to separate the polyphenols (Table 2.1). For MEKC three different pH ranges have been used namely pH lower than 8 (group 1), pH 8-9 (group 2) and pH higher than 9 (group 3). For CZE only buffers with a pH higher than 9 (group 4) have been used. In group 1, low to moderate concentration of borate was mixed with phosphate when SDS or CTAB was used as the surfactants. These methods relied mainly on the hydrophobic interaction with the surfactants for selectivity. Borate complex formation and weakly anionic properties contributed selectivity to a lesser extent. Phosphate, cholate and taurine systems have also been used where the chiral taurine adds selectivity as well. In group 2, moderate to high concentrations of borate were used with SDS as surfactant. These methods relied mainly on borate complex formation and the anionic properties of the polyphenols. Hydrophobic interactions were not as important as can be seen from the use of low SDS concentrations used in these methods. In group 3 low concentrations of borate, phosphate, SDS and SC were used. The negative charges on the polyphenols were the major selectivity factor. Borate complex

Table 2.1 The importance of pH on the selection of MEKC and CZE buffer conditions for flavonoids and flavonoid glycosides separation methods.

pH	Buffer Conditions	Surfactant	Reference
Group 1			
6.97	5mM Borate, 15mM Phosphate, 40%acetonitrile	50 mM SC	(Li, 1995)
7.5	50mM Borate, 50mM Phosphate	42mM SDS	(Ng, 1992)
7.0	12mM Borate, 20mM Phosphate, 4%1-propanol 100mM Phosphate500mM Taurine, 6% 1-propanol	40mM CTAB 35mM cholate	(Bjergegaard, 1993)
Group 2			
8.3	20mM Borate	50mM SDS	(Pietta, 1994)
8.0	200mM Borate	50mM SDS	(Ferreres, 1994)
8.0	20mM Borate, 20% Methanol	25mM SDS	(Morin, 1997)
8.0	200mM Borate, 10% Methanol 100mM Borate, 10% Methanol 200mM Borate, 10% Acetonitrile	50mM SDS 30mMSDS 50mMSDS	(Gil, 1995)
8.3	20mM Borate	50mM SDS	(Pietta, 1991)
8.5	20mM Borate, 1mM 1,3-diaminopropane	48mM SDS	(Liang, 1996)
Group 3			
9.7	12.5mM Borate, 10mM Phosphate	20mM SDS	(Liu, 1994)
9.35	12.5 mM Borate, 10 mM Phosohate	18mM SDS, 2mM SC	(Sheu, 1995)
Group 4			
9.5	25 mM Ammonium acetate	None	(Aramendia, 1995)
9.5	25mM Borate	None	(McGhie, 1993)
10.0	150 mM Borate	None	(Seitz, 1992)
9.6	100 mM Boric Acid	None	(de Simón, 1995)

formation and hydrophobic interaction added selectivity to a lesser extent. In group 4 only borate in moderate to high concentrations has been used. The charge to mass ratio differences results in separation in the CZE mode. Charges originate from the deprotonated hydroxyl groups as well as the borate complex formation that takes place.

Several properties of analytes can be exploited to achieve separation with MEKC. The analytes with hydrophobic moieties undergo hydrophobic interactions with the surfactants. Changes in the surfactant concentration will change the distribution of the hydrophobic analytes between the surfactant and aqueous phase. The addition of organic solvents will make hydrophobic analytes more soluble in the aqueous phase, thus changing the distribution pattern. The selectivity can also be manipulated when chiral selectors are added to the aqueous phase. These chiral selectors form complexes with the analytes in the aqueous phase. This changes the distribution between the surfactant and aqueous phase. Borate is one chiral selector normally used to obtain separation of polyphenols. It complexes only with vicinal hydroxyl groups on sugars or aromatic rings. The negatively charged borate then results that the analyte is more negatively charged. This prevents the analyte from entering the surfactant phase. It also changes the charge/mass ratio and migration properties of the analyte. Changes in the pH of the aqueous phase, especially near the pKa values of analytes are useful in changing the migration properties for achieving separation.

Our aim was to develop one method to analyze tea catechins (weakly anionic), caffeine (neutral), theanine (zwitterionic) and ascorbic acid and gallic acid (anionic). Caffeine was found to co-migrate with the electro-osmotic flow (EOF) in a buffer

lacking surfactants (CZE), with pH 5-8 being tested (Lee, 1992). In a buffer lacking surfactant the zwitterionic theanine will also co-migrate with the EOF. The lack of selectivity by ITP and CZE for neutral and zwitterionic compounds ruled them out as options for method development. MEKC was deemed the best CE mode to separate the weakly anionic, anionic, neutral and zwitterionic compounds of interest. Since the majority of the publications used MEKC successfully for polyphenol separations, it seemed likely that success would be possible with this mode of CE.

Besides our MEKC method, eight other CE methods exist to analyze different tea polyphenols with, one CZE method for the analysis of organic anions in tea (Horie, 1998 (a)) and one cLC/ESI-MS method for the detection of catechins in tea and in human plasma (Dalluge, 1997). Of the eight methods on catechins, two of them are CZE methods. Boric acid was used to separate the five major catechins, quercetin, gallic acid, caffeic acid, theophylline and adenine (Arce, 1998). Borate was used to separate the five major catechins as well as caffeine, theanine and ascorbic acid (Horie, 1997). The conditions used in this method lead to significant peak tailing of all the analytes, resulting in inaccurate quantification. This is probably why the same author developed MEKC methods to separate the same analytes (Horie, 1998 (b)), (Horie, 1999). Some of the catechins still showed significant peak tailing. Two other methods were developed that used the combination of SDS, sodium tetraborate and sodium phosphate to separate the tea catechins (Watanabe, 1998), (Barroso, 1999). A MEKC method using borate, SDS and β-cyclodextrin was developed by (Nelson, 1998) to separate six tea catechins. A MEKC method was developed that used borate complexing and acetonitrile organic phase partitioning at pH 6.0 (Larger, 1998). This method was used to analyze catechins, flavonoid glycosides and chlorogenic acid ob-

tained with aqueous, ethyl acetate or methanol extraction procedures. Their results give an appreciation for the complexity of tea samples. It is unlikely that one single set of analytical conditions will be sufficient to analyze all the diverse compounds in tea infusions e.g. catechins, theaflavins, thearubigens, flavonoid glycosides, carboxylic acids, amino acids and carotenoids.

2 Materials and Methods

2.1 Strategy for optimization of a MEKC method

Many molecules can behave as acids or bases. The pH of the buffer has a major influence on the selectivity. The pH conditions should be considered well, since pH influences the EOF and determine the charge to mass ratio of the analytes. For the analysis of small molecules it is best to select the pH conditions in the range of the pKa values of the analytes, since the most selectivity can be achieved then (Wätzig, 1998). From Table 2.1 it is clear that pH is the most important parameter in this situation even though a MEKC method (with predominantly hydrophobic interactions) is being developed. The pH value determines the type of buffer and the physiochemical properties that will be exploited to achieve separation. Several preliminary studies were conducted. The pH was tested in the range of 7-8 and it was found that the pH should be kept below 7.6 to prevent peak shape distortion. This restriction places the method in group 1 with borate/phosphate mixtures and SDS or CTAB as surfactants. Another preliminary study showed that the borate caused severe peak tailing whereas phosphate provides more symmetrical peaks. It was decided to optimize a method with phosphate and SDS in the pH range of 6.5 to 7.6. Methods can be developed with either the overlapping resolution mapping (ORM) procedure or with the simple uni-

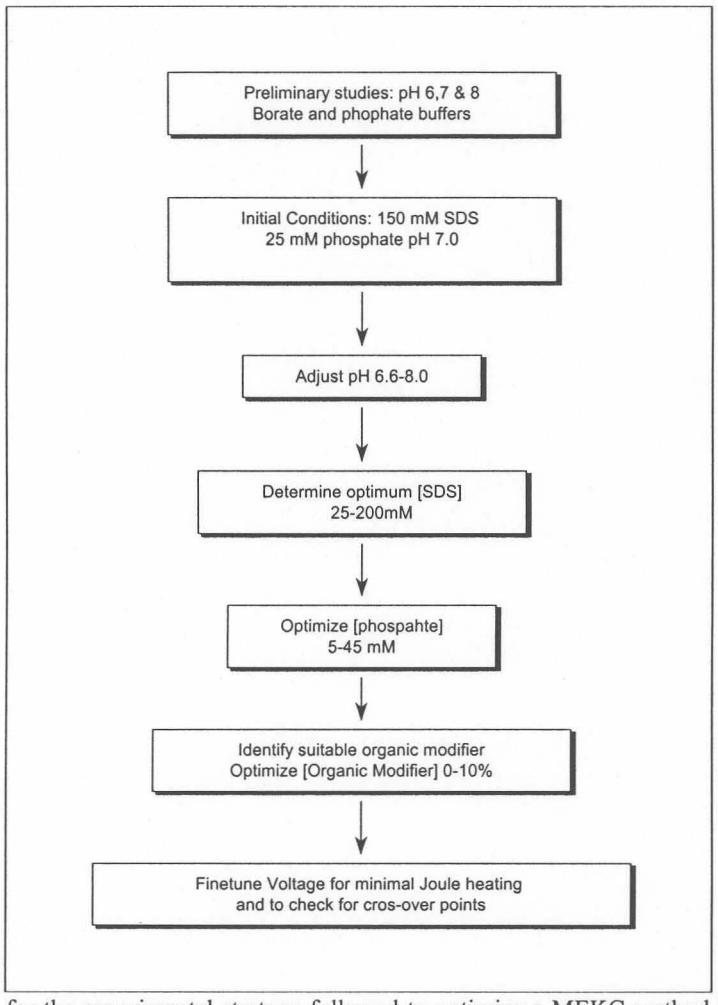

Figure 2.1 Scheme for the experimental strategy followed to optimize a MEKC method to analyze tea catechins.

variate procedure. With the ORM procedure two or three parameters can be varied at the same time (Ng, 1992). Polynomial equations are then derived to define the resolution between neighboring peaks. A global optimum condition is then derived. This procedure results in optimization with typically 9 runs or less. Unfortunately the migration properties of the analytes are defined theoretically and knowledge of precise crossover regions and deviations from the theoretical models are not available. We opted for the univariate procedure where only one parameter is varied at a time. This enabled us to define the migration behaviors of all the analytes and select conditions far from regions that may result in inconsistent separations. The univariate

procedure also allows easier fine-tuning of the method to reduce analysis times to a minimum. A flow diagram of the optimization procedure is depicted in Fig. 2.1.

2.2 Instrumentation

Electrophoresis was carried out using the Beckman P/ACE 2100 (Beckman Instruments, Fullerton, CA, USA) with on-column detection. An uncoated fused silica capillary column from Beckman with an internal diameter of 50 μm and total length of 57 cm was used. The effective separation length was 50 cm. All samples were injected pneumatically (0.5 psi) for 2 sec. The operating temperature was 25°C. Detection was effected by measurement of UV absorbance at 200 nm.

2.3 Reagents

The five catechin standards (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg) and (-)-epigallocatechin gallate (EGCg) as well as theanine were gifts from Mutsui Norin (Tokyo, Japan). The gallic acid and ascorbic acid were obtained from Sigma Chemical Company (St Louis, MO, USA). Caffeine and sodiumdodecyl sulphate (SDS) were obtained from Merck (Darmstadt, Germany). The SDS was of analytical grade suitable for electrophoresis. All buffer salts were of analytical grade. All solutions were prepared with distilled water that was deionized with a Milli-Q system (Millipore Corp., Bedford, MA., USA).

Methanol and *p*-nitrophenol were obtained from BDH Laboratory Supplies (Poole, England) and Sudan III from SAARChem Pty. Ltd. (Muldersdrift, RSA). The metha-

nol and Sudan III were used for the capacity factor (k') calculations of each analyte. *p*-Nitrophenol was used both as reference peak and internal standard.

2.4 Analytical conditions

Standards and samples were analyzed with the anode on the inlet side and a running buffer of 100 mM SDS, 25 mM phosphate, 6% (v/v) methanol pH 7.0. The buffer pH was adjusted by mixing monosodium and disodium phosphate (25 mM) solutions. Samples of fresh tea leaves as well as green and black tea were analyzed with an applied voltage of 14 kV. Bottled tea samples were analyzed with the running buffer containing only 5% (v/v) methanol and an applied voltage of 14 kV.

At the beginning of each day the capillary was regenerated by rinsing for 5 min with H_2O, 10 min with 0.1M HCl, 5 min with H_2O, 5 min with 1.0M NaOH, 5 min with H_2O, 10 min with 0.1M NaOH and finally 2 min with H_2O. The column was equilibrated by repeating 5 analyses of the standards after the regeneration. Before each analysis the capillary was rinsed with running buffer for 2 min. After each analysis the capillary was rinsed for 30 sec with H_2O, 2 min with 0.1 M NaOH and 2 min H_2O.

2.5 Preparation of samples and standard

Fresh tea leaves were obtained from the Grenshoek tea estate in the district of Tzaneen in South Africa. Black tea was prepared in our laboratory. Briefly the leaves were withered overnight at room temperature, macerated by three passes through a cut, tear and curl (CTC) machine and left at room temperature for 60 minutes to oxidized. The oxidized leaves were dried in a fluid bed drier. The dried tea was sifted

in a series of sieves and only particles between 500 and 1700 µm were selected for further work. Green tea was prepared in the same manner with omission of the oxidation step. All samples were stored in airtight aluminium lined paper bags at 4°C.

Fresh tea leaves were steamed for 30 seconds and oven dried at 90°C overnight. Bottled ice teas were obtained from a local supermarket. Samples were not filtered with membrane or cartridge filters as some polyphenols absorb to the filter material.

Black and green tea liquors were prepared from made tea samples. Tea liquors were prepared as 1% (w/v) tea solutions (1 g dry, fine ground tea leaves in 100 ml H_2O). The water extracts of tea leaves were made by shaking the tea leaves for 10 min in boiling hot H_2O in a thermal flask. The extracts were then filtered through Schleicher & Schüll No. 595 filter paper (Germany) to remove particulate matter. This is the concentration of tea normally consumed by the general public. Professional tea tasters use a 2% (w/v) concentration for easier detection of off-notes and deleterious flavours. Filtered black tea samples were diluted to 0.5% (w/v), while filtered green tea samples were diluted to 0.1% and 0.5% (w/v) with 0.1mg/mL pNP dissolved in H_20 before injection. pNP was added to bottled tea at a concentration of 0.1mg/ml before analysis.

All standards were prepared by dissolving individually in H_2O. EC and ECg were prepared at 0.5 mg/ml, theanine and ascorbic acid at 10 mg/ml, pNP at 5 mg/ml and all the other analytes at 1 mg/ml.

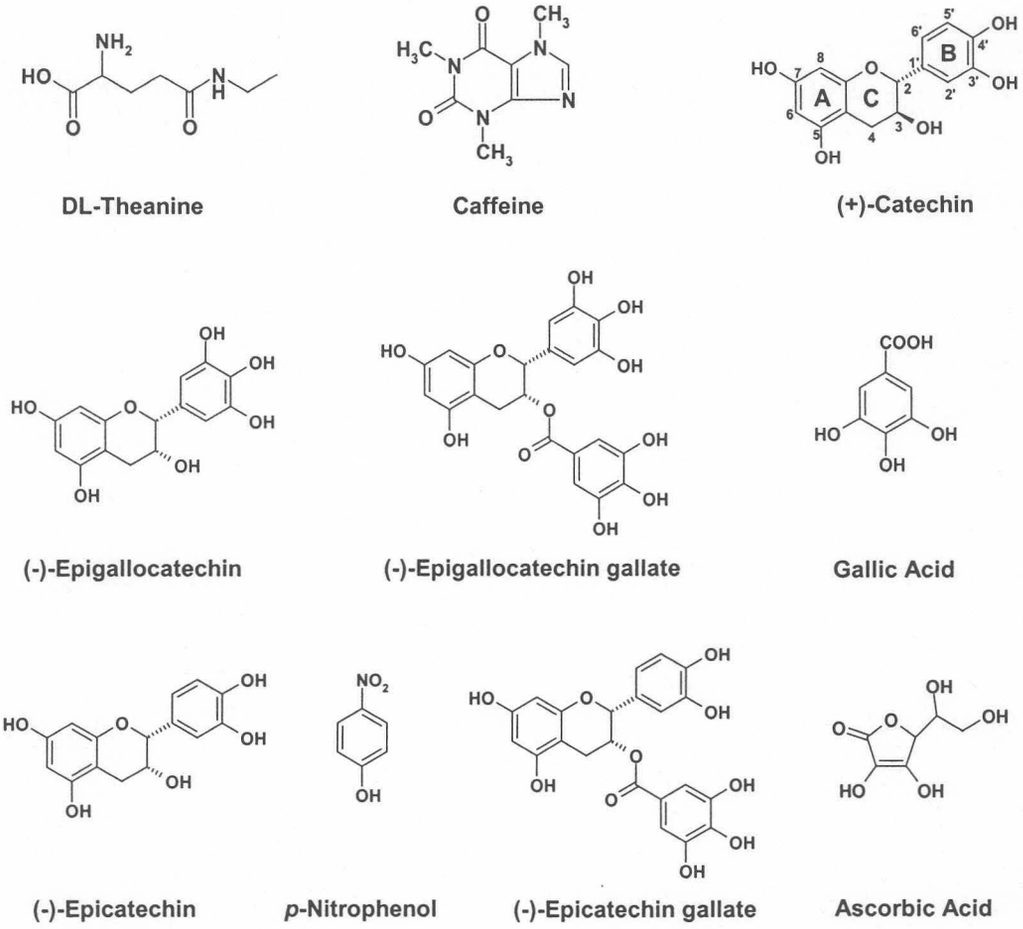

Figure 2.2 Chemical structures of organoleptically important compounds in tea and the pNP internal standard.

3 Results and Discussion

The compounds that have a large influence on the quality of tea were used in this investigation (Fig. 2.2). The influence of pH, surfactant concentration, background electrolyte, organic modifier and voltage on their resolution, peak shape and capacity ratios were determined. Near optimum conditions were selected from our experience. Each variable was changed individually over the range of interest while all other variables were kept constant. All optimization analyses were done at an applied voltage of 14 kV.

Capacity factors (k') were calculated with equation 1 and used as a convenient way of expressing the results. All migration times were measured in minutes.

$$k' = \frac{t_r - t_0}{t_0(1 - \frac{t_r}{t_{mc}})} \qquad (1)$$

k' = capacity factor

t_0 = retention time for unretained compound

t_r = retention time of analyte

t_{mc} = retention time of completely retained compound

The values t_o and t_{mc} were determined with methanol and Sudan III respectively. These values are indicative of the electro-osmotic flow (t_o) and surfactant phase (t_{mc}) velocities.

The column efficiency (N) was calculated with equation 2 and the resolution (R_s) between peaks with equation 3. In equation 3 t_{r1} and t_{r2} are the migration times of two neighboring peaks, where t_{r2} has the longer migration time. The symbols w_1 and w_2 represent the peak widths at baseline that correspond with t_{r1} and t_{r2} respectively.

$$N = \frac{(t_r)^2}{\left(\frac{w}{4}\right)^2} \qquad (2)$$

$$R_s = \frac{(t_{r2} - t_{r1})}{\frac{1}{2}(w_1 + w_2)} \qquad (3)$$

3.1 Influence of pH

The pKa values of the catechins are not listed in the literature. The pKa values of the pyrocatechol and pyrogallol subunits are 9.37 and 9.28 respectively (Nelson, 1998) The presence of the electron-withdrawing aromatic rings will tend to decrease the pKa values further. It is safe to assume that the polyphenols have pKa values between 8 and 10. This causes them to be neutral to partially anionic in the tested pH range 6.6 to 8.0. A running buffer with 150 mM SDS and 25 mM sodium phosphate was used for the pH optimum study. The pH was adjusted by changing the ratio of mono- and disodium phosphate in the buffer. The EOF did not change significantly in this pH range. All changes in the separation of the analytes are due to the changes in their anionic charges. This caused changes in the distribution coefficient of the analytes between the bulk and micellar phases. The decrease in pH had the most significant effect on the capacity factors (k') of the more hydrophobic ECg and changed the k' of less hydrophobic C, EC, EGC and EGCg only slightly (Fig. 2.3). No significant changes in k' of caffeine, ascorbic acid, gallic acid, theanine and pNP were found in this pH range. This is because none of these analytes have pKa values in the tested range.

The peak shapes of EGC, EGCg, ECg were affected significantly by increase in pH. The polyphenols showed extensive peak tailing and distortion above pH 7.6 (results not shown). There can be several reasons for this behavior. This could be the result of

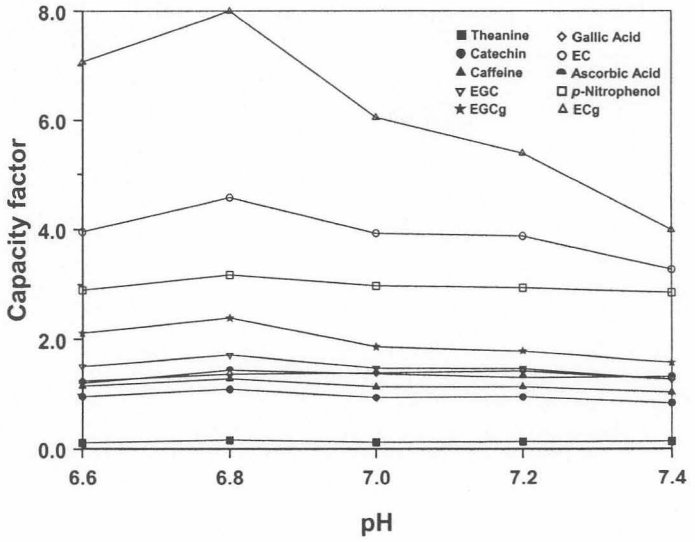

Figure 2.3 Influence of pH on the capacity factors of compounds. Conditions: 150 mM SDS, 25 mM phosphate buffer, 0% (v/v) methanol. Separation with 14 kV.

auto-oxidation of the catechins in an alkaline environment. Alternatively the polyphenols become more negatively charged, resulting in unwanted interactions with the column or the surfactant. Ascorbic acid showed an increased leading front with a decrease in pH. The peak shape of gallic acid, caffeine, theanine and pNP were unaffected by pH changes in this range. The optimum pH was chosen as pH 7.0 because peak shapes did not improve with further decrease in pH. Lower pH only increased the analysis time and therefore the capacity factors but not the resolution of the analytes. Generally capacity factors between 0.5 and 5 are required for good separation. With the running buffer conditions as mentioned above, only gallic acid and ascorbic acid co-migrates at pH 7.0 (Fig. 2.3).

3.2 Influence of SDS concentration

An increase in surfactant concentration, and thus micellar-phase volume, showed an increase in the k' of all analytes except for theanine, gallic acid and ascorbic acid. These compounds were not influenced significantly (Fig. 2.4). They had a higher affinity for the bulk phase than for the micellar phase and their ionic properties influenced their separation. The negatively charged ascorbic acid and gallic acid are the least soluble in the negatively charged SDS pseudo-stationary phase due to electro-

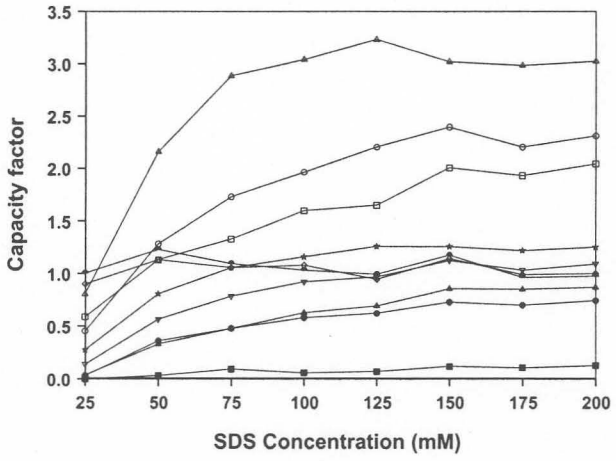

Figure 2.4 Influence of SDS concentration on the capacity factors of compounds. Symbols as in Fig. 2.3. Conditions: 25 mM phosphate pH 7.0, 0% (v/v) methanol. Separation with 14 kV.

static repulsion, yet their $k' \approx 1$. This is because these organic acid anions have a negative migration mobility against the EOF resulting in a longer migration time than t_0. Theanine, a zwitterion with an overall charge of zero, elutes very early with $k' \approx$ 0.1. This is probably because theanine is retained only slightly when the hydrocarbon side-chain and the α-amino group form weak interactions with the surfactant. The hydrophobicity of ECg caused its k' to increase drastically with increasing SDS concentration. The linear relation of k' vs. [SDS] for the catechins and caffeine (Fig. 2.5)

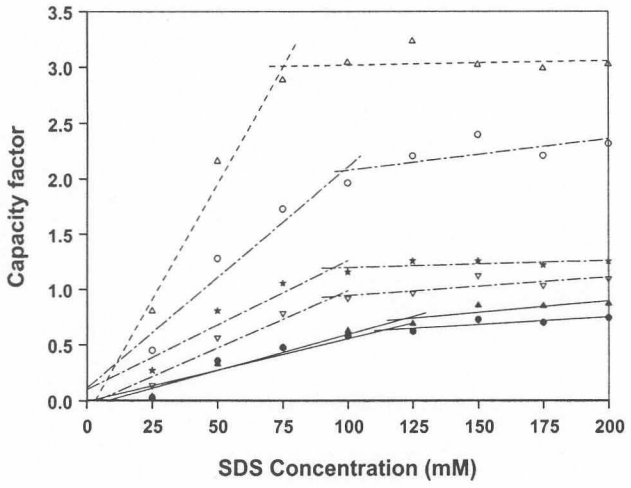

Figure 2.5 Linear dependence of analyte capacity factors on the surfactant concentration. The symbols represent the same analytes as in Fig. 2.3 and the conditions are the same as in Fig. 2.4.

is an indication that there are no significant charges on the catechins (Terabe, 1985). The least hydrophobic analytes (caffeine and catechin) display a first order linearity up to 125 mM, the more hydrophobic EGC, EGCg and EC display a first order linear dependence up to 100 mM and he most hydrophobic catechin, ECg, only up to 75 mM SDS. After the first order linear region follows a zero order linear region indicating that an equilibration or saturation point is reached where the catechins do not show an increased uptake into the micelles. All of them intercept in the vicinity of the origin, indicating their dependence on the surfactant concentration.

An increase in the SDS concentration extended the retention window (Fig. 2.6) and helped to resolve the different analytes. The migration window did not become larger with increase in SDS concentration above 150 mM. The concentration of 100 mM SDS provided good resolution of almost all the compounds (Fig. 2.4). Fig. 2.7 shows the effect of increased SDS concentration on the platenumbers for caffeine, EC and pNP. The platenumbers increase up to 100 mM SDS, as the effects of micelle poly-

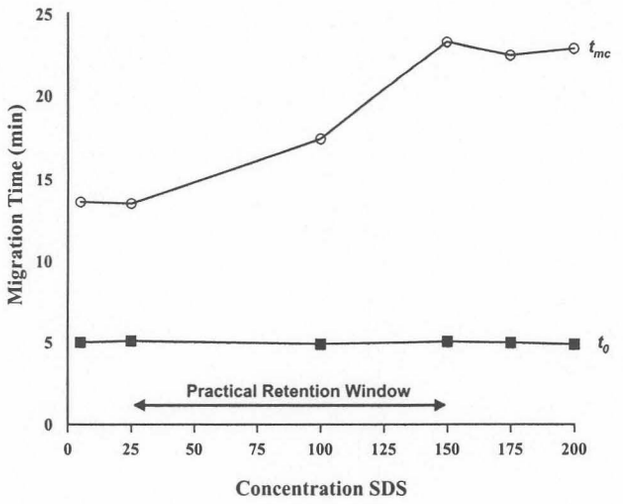

Figure 2.6 The effect of the surfactant concentration on the retention window required for separation of analytes. The increase in SDS concentration had no effect on the migration time of the EOF (t_0) (■). The migration time of the surfactant phase (t_{mc})(O) showed a first order linear relationship with increase of SDS from 5 mM-150 mM. There was a zero order linear relationship between the surfactant phase migration time and the SDS concentration between 150 mM and 200 mM.

dispersity are reduced. An increase in surfactant concentration reduces polydispersity. Polydispersity is the variation in aggregation number of the micelles, resulting in micellar heterogeneity. As the surfactant concentration increases, the intermicelle mass transfer and monomer/micelle exchange rates increase. This results in less variation in the micellar aggregation number and ensures more homogenous micellar structures. The maximum in platenumbers is achieved in the concentration range of 100-125 mM SDS. A further increase in surfactant concentration results in decreased platenumbers due to thermal band dispersion. The thermal band dispersion is a result of the high conductivity and excessive Joule heating.

Black tea contains a diverse group of catechin dimers (bisflavanols and theaflavins) and polymers (thearubigens). The rather high SDS concentration results in slower

migration times for these more hydrophobic multimeric forms than for the monomers. A clean and stable baseline is obtained for the region of importance, resulting in easier detection of the catechins. After taking all the data into consideration it was decided to use 100 mM SDS in the following steps of the univariate procedure.

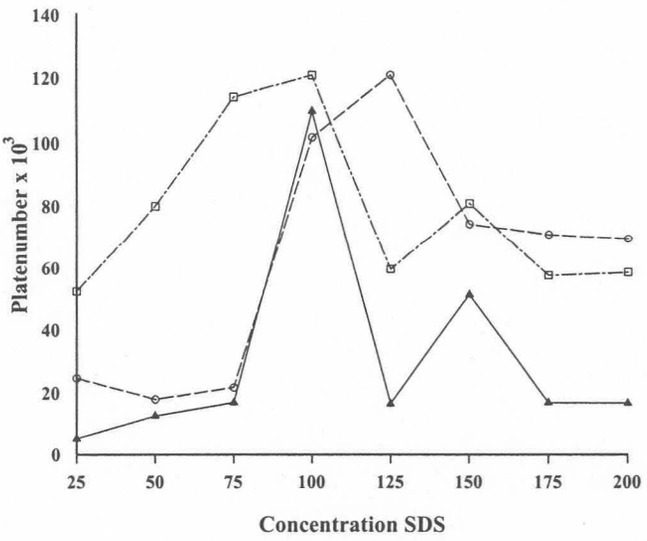

Figure 2.7 The effects of increased surfactant concentration on the column efficiency (N) as calculated for the caffeine (▲), EC (O), and pNP (□) peaks.

3.3 Influence of phosphate concentration

An increase in the concentration of the background electrolyte (BGE) resulted in a decrease in the EOF. This is consistent with results found by other researchers who found a linear relationship between the mobility of the EOF and the natural logarithm of the concentration anions in the running buffer (VanOrman, 1991). The *k'* decreased with an increase in the BGE concentration (Fig. 2.8). The increased ion concentration resulted in higher conductivity and Joule heating. The temperature of the running buffer could increase with excessive Joule heating. An increase in temperature can shift the phase-partitioning coefficients more to the aqueous phase with the hydro-

phobic compounds becoming more soluble in the water. This could decrease the capacity factors despite of a decrease in the EOF. Increased BGE concentration resulted

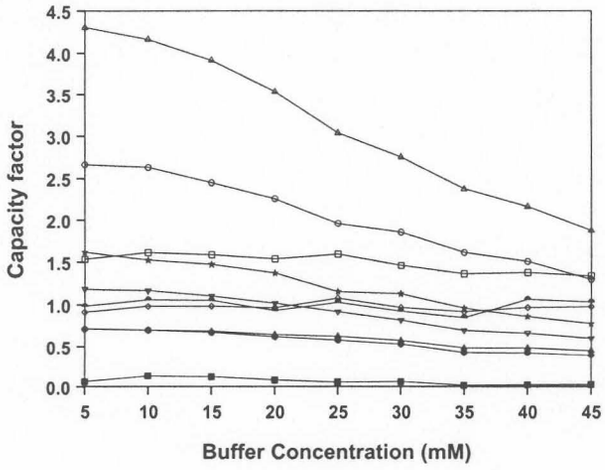

Figure 2.8 Influence of background electrolyte concentration on the capacity factors of compounds. Symbols as in Fig. 2.3. Conditions: 100 mM SDS, pH 7.0, 0% (v/v) methanol. Separation with 14 kV.

in a significant increase in the sharpness and resolution of the peaks. This is the result of stacking, which occurs when the buffer zone has a higher conductivity than the sample zone. The field strength is higher in the sample zone causing a concentration effect of the charged analytes (Chien, 1992). The phosphate and sodium ions in the running buffer bind to the silica column. When these ions shield the hydroxyl groups of the column it is called screening. By screening the column the zeta potential of the inner surface is reduced and thus also the EOF. At high ion concentrations this screening becomes less reproducible and fluctuation in the EOF and analyte migration times occur more frequently. The phosphate ions do not affect the micellar phase-bulk phase partitioning, thus having no contribution to the selectivity of the catechins. A concentration of 25 mM phosphate was considered as optimal. The peaks were sharp, the migration times were reproducible and the Joule heating was within the limits when separation was done at 14 kV.

3.4 Influence of organic modifier concentration

The effects that acetonitrile, methanol and 1-propanol have on the separation of the catechins were determined. The organic modifiers were tested in the range of 0-10% (v/v). Methanol was selected as organic modifier, since it increased the selectivity of the buffer, separating the analytes well. The effects of the methanol concentration on the capacity factors are shown in Fig. 2.9. An increase in methanol increased the t_0

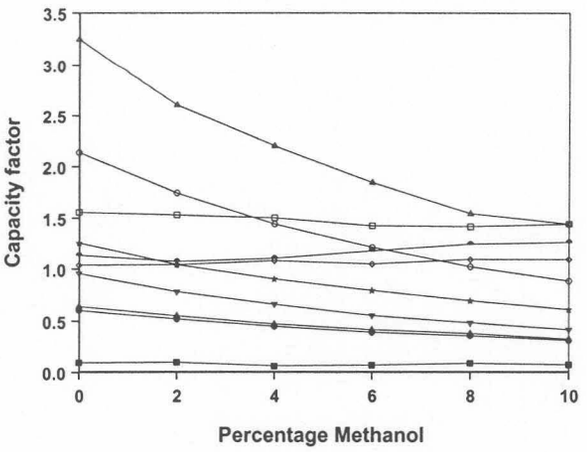

Figure 2.9 Influence of organic modifier on the capacity factors of compounds. Symbols as in Fig. 2.3. Conditions: 100 mM SDS, 25 mM phosphate, pH 7.0. Separation with 14 kV.

and t_{mc} values. A linear quantitative relationship between $1/k'$ and the concentration organic modifier was derived by (Chen, 1995 (a)). The linear relationship is only observed when no significant change occurs on micellar structure upon the addition of the organic modifier. When the organic modifier concentration is to high, deviations from the straight line is observed since the ionic conditions and the aggregation number of the micelles change. The relationship between $1/k'$ and percentage methanol was linear (Fig. 2.10A), indicating that there was no change in micellar structure upon addition of the organic modifier. The plot of mobility ratio vs. organic modifier (Fig. 2.10B) gives the same conclusion. The mobility ratio mainly characterizes the surface

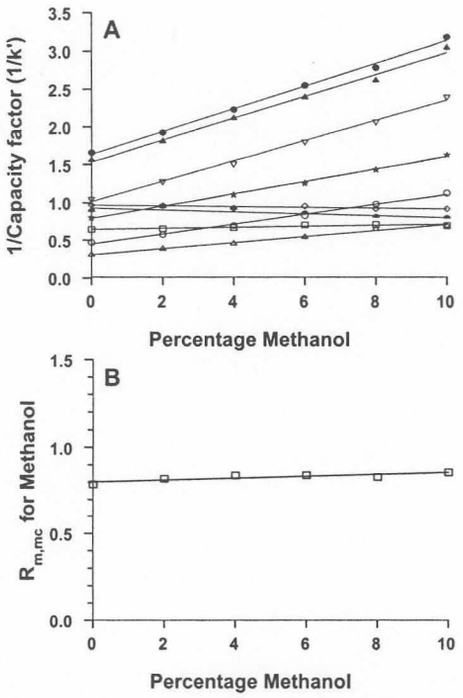

Figure 2.10 The linear quantitative relationships found for **A**:1/*k'* vs. methanol concentration and **B**: $R_{m,mc}$ vs. methanol concentration. This indicates that the methanol had no effects on the micellar structure or surface charges in the range tested. The symbols for Fig 2.10A is the same as for Fig 2.3 and the conditions are the same as in Fig. 2.9.

charge density of the micelles and is given by the equation $R_{m,mc} = 1 - {}^{t_o}/_{t_{mc}}$. It was found to be a good parameter in the investigation of the effect of organic modifier on the micelles. A linear relationship can generally be found between $R_{m,mc}$ and low concentrations of organic modifier where the CMC of the surfactant is not influenced (Chen, 1995 (b)). From this it can be concluded that all changes in *k'* could be contributed to changes in the bulk phase-surfactant phase distribution coefficients. The *k'* of the relatively more hydrophobic compounds (EC, EGCg and ECg) decreased most significantly with an increase in percentage methanol. This is an indication that the compounds become more soluble in the bulk phase because they are more soluble in methanol. The methanol does not alter the solubility of the ionic

analytes (theanine, gallic acid and ascorbic acid) in the bulk phase resulting in no changes in their *k'* values. A 6% (v/v) methanol concentration was considered optimal

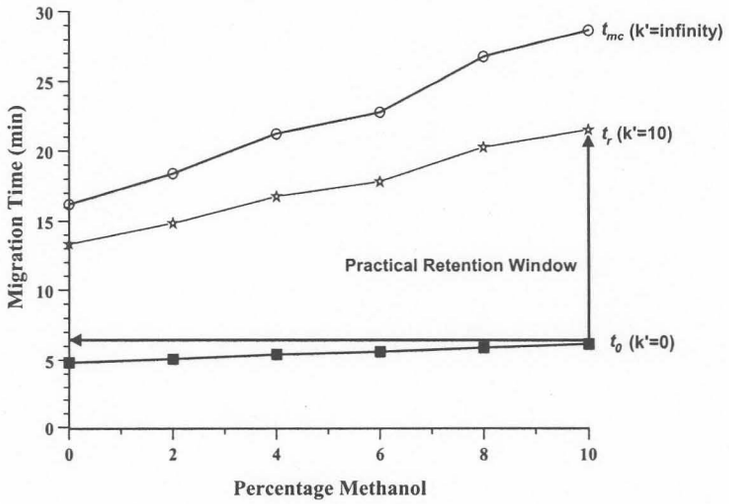

Figure 2.11 The effects of the methanol concentration on the retention window required for separating the analytes. The increase in methanol concentration increased the migration time EOF (■) slightly. The migration time of the surfactant phase (O) showed a first order linear relationship with increase of methanol from 0%-10% (v/v).

for the analysis of green and black tea samples because it provided the shortest analysis time with good resolution. Bottled tea samples required an optimal concentration of 5% (v/v) methanol to provide good resolution between ascorbic acid and EC. Methanol concentrations of 5 and 6% (v/v) provide a window of more than 15 minutes (between the EOF and t_{mc}) for all the 9 analytes to elute in (Fig. 2.11). Since the micellar phase is moving, compounds with a $k' = \infty$ will also move through the column. This results that analytes with *k'* between 10 and ∞ are crammed together in the last part of the electropherogram. From a practical point of view it is best if the analyte migrating last has a *k'* of no more than 10. This ensures that the analyte peaks are evenly spaced. When taking this into consideration the retention window is reduced to 12.20 min.

The tables of organic solvent properties (Snyder, 1978) show that the dipole strength decrease in the order acetonitrile > methanol > 1-propanol. From this it can be concluded that acetonitrile is the most polar and 1-propanol the least polar of the three solvents. The table for the solvent strengths indicate that 1-propanol is the strongest solvent, while methanol and acetonitrile are approximately equally strong. Fig. 2.12 shows the effects of the concentration 1-propanol on the migration times of the analytes. The 1-propanol dissolves the hydrophobic catechins (EC, ECg and EGCg) more readily in the aqueous phase resulting that their phase equilibrium shifts more to

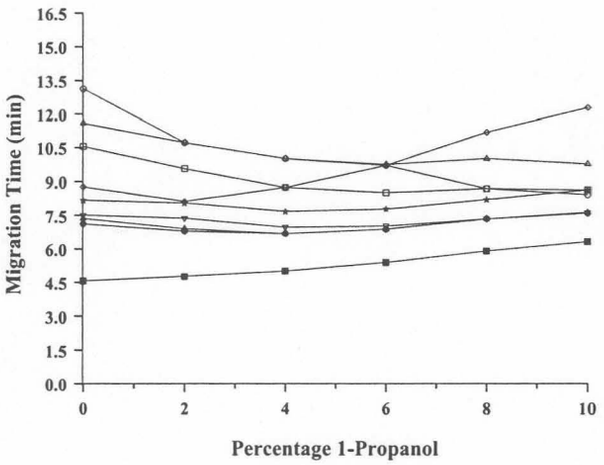

Figure 2.12 The influence of the 1-propanol concentration on the migration times of the analytes. Symbols as in Fig. 2.3. Conditions: 25 mM phosphate pH 7.2, 150 mM SDS. Separation with 14 kV.

the bulk (aqueous) phase. This explains why the more hydrophobic analytes (EC, ECg and EGCg) start to co-migrate. All conditions being the same, except for a pH of 7.2, 6% (v/v) acetonitrile did not provide the resolution obtained with 6% (v/v) methanol (Fig. 2.13). The acetonitrile is more polar than the methanol and does not effect as large a change in the phase equilibrium of EC, ECg and EGCg. The longer migration time of ECg with acetonitrile ($t_r \approx 15$ min) than with methanol ($t_r \approx 11$ min) is an indication of the effect the organic solvents have on the phase equilibrium.

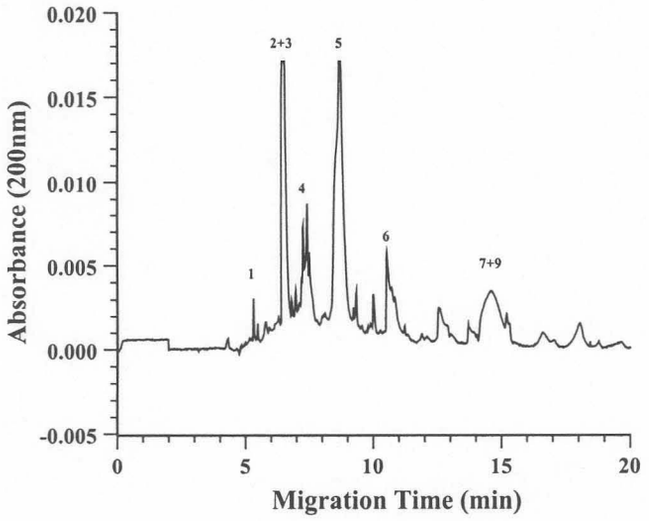

Figure 2.13 The analysis of a 2% (w/v) water extract of green tea with acetonitrile as organic modifier. Conditions: 25 mM phosphate pH 7.2, 100 mM SDS, 6% (v/v) acetonitrile. Separation 14 kV. The peaks are numbered the same as for the standards in Fig. 2.15A.

3.5 Influence of applied voltage

The applied voltage was varied from 10-20 kV. The influence of kV on *k'* is shown in Fig. 2.14. The *k'* of the zwitterion, theanine, and the anions, gallic and ascorbic acid, were effected very slightly with higher voltages. The *k'* of the caffeine, pNP and all the catechins decreased with higher voltage. The more hydrophobic the analyte is, the more extensive was the decrease in the *k'*. The increase in voltage increased the Joule heating. The increased Joule heating could increase the effective temperature in the column, thus reducing the viscosity of the buffer. This causes the micelles to move more rapidly through the column and could explain why the hydrophobic analytes were affected the most. The peaks became narrower with increasing kV, due to shorter migration times and less diffusion as can be expected. The increased Joule

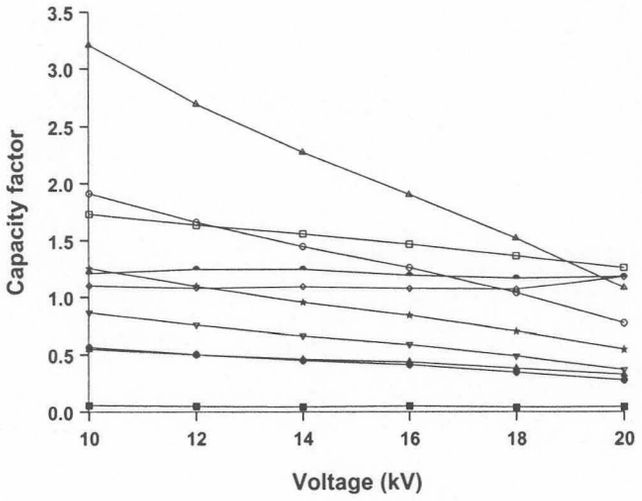

Figure 2.14 The influence of applied voltage on the capacity ratios of the analytes. Symbols as in Fig. 2.3. Conditions: 100 mM SDS, 25 mM phosphate, pH 7.0, methanol 5% (v/v).

heating results in practical problems, such as fluctuating migration times and peaks changing positions near the crossover points. The optimal voltage was selected as 14 kV because it was away from any cross-over points (near 12 and 17 kV) and the Joule heating was within the limits for the type and length of capillary that was used.

3.6 Suitability of internal standard

p-Nitrophenol (pNP) seems to have been a good selection as internal standard. The k' of pNP was not influenced significantly by changes in pH, phosphate, methanol and voltage conditions over the ranges investigated here. pNP never occurs in tea. It was used as internal standard to compensate for variation in injection volume. pNP was also used as a migration time reference compound for automated peak identification by the data system software. It migrates far from adjacent catechins, permitting a wide window definition.

3.7 Regression Analysis

The LOD's were determined and are shown in Table 2.2. The limits of quantification (LOQ) are taken as the concentrations that result in 5% or less relative standard deviation (RSD) upon quantification and meet the requirements for analysis of fresh leaf, green and black tea liquor as well as bottled ice teas. The upper limit of linearity for calibration curves were determined and calibration curve correlation coefficients (r) were better than 0.994. All results are shown in Table 2.2.

Table 2.2 Limits of detection and quantification values, linear limits and regression coefficients of all analytes.

Compound	LOD (µg/ml)	LOQ (µg/ml)	Linear Limit (µg/ml)	r
Theanine	4.0	10.0	600.0	0.995
Caffeine	2.0	5.0	75.0	0.998
Catechin	1.0	2.0	55.0	0.999
EGC	2.0	4.0	55.0	0.997
EGCg	6.0	11.0	110.0	0.994
Gallic Acid	2.5	6.0	120.0	0.998
EC	1.0	2.0	135.0	0.996
pNP	4.0	8.0	160.0	0.998
ECg	3.0	10.0	100.0	0.994
Ascorbic Acid	20.0	50.0	500.0	0.994

3.8 Validation of analytical procedure

Precision tests were done to determine both intra-day and inter-day variation in migration times. Calculations are based on the analysis of green tea samples. The gallic and ascorbic acid levels in green tea are below the LOD and therefore no data is available for gallic and ascorbic acids. Table 2.3 summarizes the statistical evaluation

of data from 32 consecutive runs for the intra-day precision test and data from 9 days with a minimum of 10 runs per day for the inter-day precision test. The compounds with the longest migration time showed the largest standard deviation (S.D.) and %RSD. From this one would expect gallic acid and ascorbic acid to have %RSD values higher than that of EGCg and less than that of EC.

Table 2.3 Statistical evaluation of intra-day and inter-day data to determine the variation in the migration times. (MT = migration times in minutes). The analytical conditions are the same as in Fig 2.15.

Compound	Intra-day MT n=32		Inter-day MT n=9	
	MT	%RSD	MT	%RSD
Theanine	6.29	2.10	6.33	4.70
Catechin	7.29	2.24	7.37	3.65
Caffeine	7.43	2.15	7.49	3.28
EGC	7.84	2.38	8.00	3.33
EGCg	8.67	2.52	8.79	3.81
Gallic Acid	-	-	-	-
Ascorbic Acid	-	-	-	-
EC	9.84	2.67	10.03	3.91
pNP	10.74	2.47	10.84	3.72
ECg	11.39	3.02	11.59	4.22

Repetitive runs were done with green, black and bottled teas to determine the repro-ducibility of both the extraction and analysis of the samples. The same green or black tea was extracted five separate times and each infusion was analyzed in five-fold. An electropherogram of the standards is shown in Fig. 2.15A while green and black tea profiles are shown in Fig. 2.15B and 2.15C respectively. Five spiked solutions were

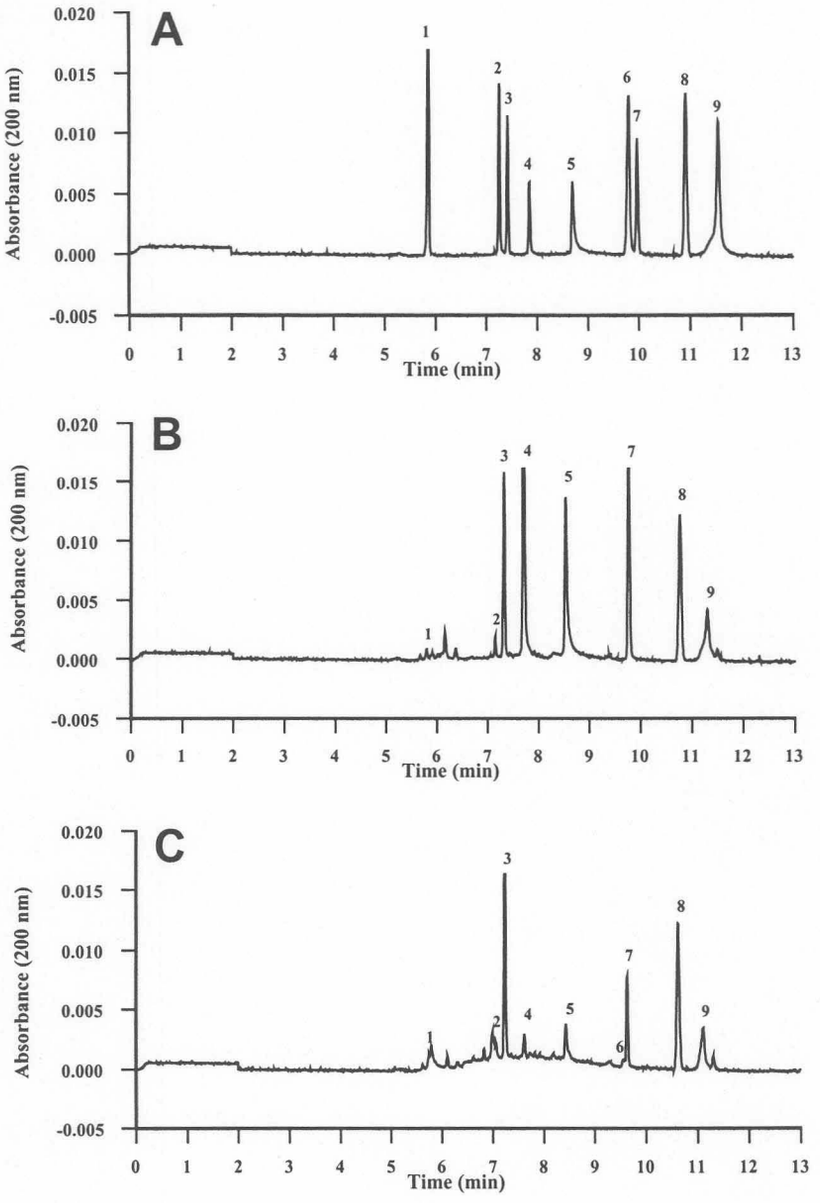

Figure 2.15 **A**: Separation of standard mix. The peaks are: 1 theanine, 2 catechin, 3 caffeine, 4 EGC, 5 EGCg, 6 gallic acid, 7 EC, 8 pNP, 9 ECg. **B**: Separation of fresh leaf sample 1% (w/v) solution 5 times diluted. **C**: Separation of black tea sample 1% (w/v) solution 3 times diluted. Conditions: 100 mM SDS, 25 mM phosphate, 6% (v/v) methanol pH 7.0. Separation was done with 14 kV. Detection at 200 nm.

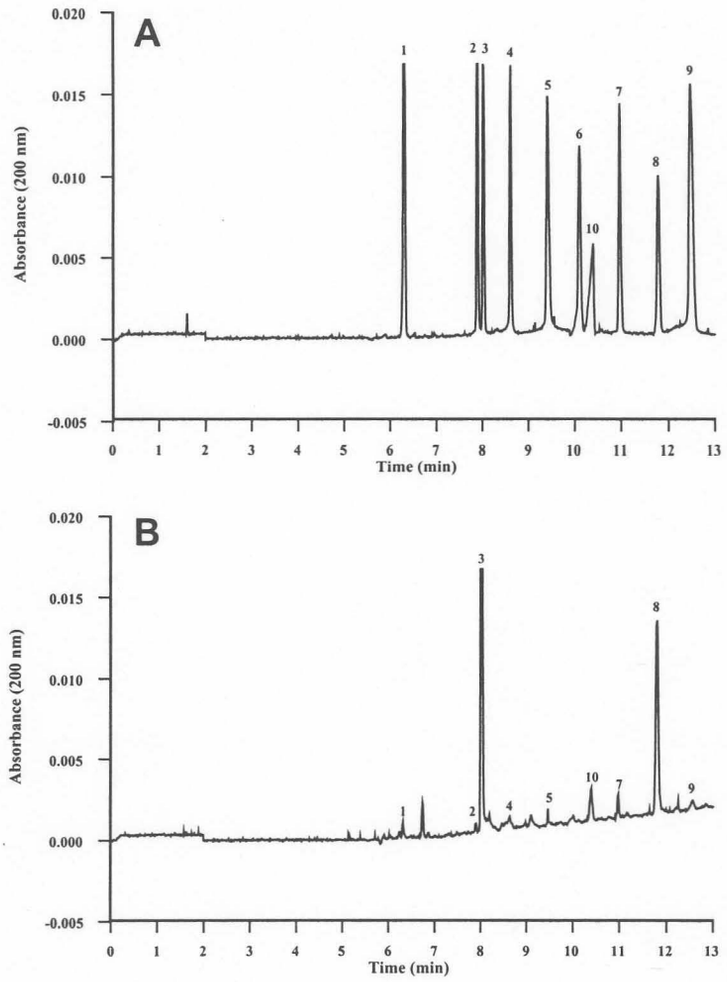

Figure 2.16 **A**: Separation of standard mix. All peaks are the same as in Fig. 2.15A and peak 10 is ascorbic acid. **B**: Separation of undiluted canned tea sample. Conditions: 100 mM SDS, 25 mM phosphate, pH 7.0, 5% (v/v) methanol. Separation was done with 14 kV. Detection at 200 nm.

prepared from one sample of bottled tea and analyzed in five-fold. The results are shown in Table 2.4. Typical electropherograms of the standards (including ascorbic acid) and a sample of bottled black iced tea are shown in Fig. 2.16A and 2.16B respectively.

Table 2.4 Typical concentrations of analytes found in dried fresh leaf (green tea), black tea (mg/g dry weight) and bottled tea (μg/ml). A typical cup of tea (black or green) is brewed as a 1% (w/v) solution.

Compound	Dried Fresh Tea Leaf		Black Tea		Bottled Black Tea	
	mg/g	%RSD	mg/g	%RSD	μg/mL	%RSD
Theanine	11.15	5.45	7.27	4.42	< LOQ	-
Caffeine	37.93	1.18	20.59	3.80	100.33	4.07
Ascorbic Acid	< LOD	-	< LOD	-	224.98	4.60
Gallic Acid	< LOQ	-	2.29	2.33	< LOD	-
Catechin	0.87	3.71	1.21	4.92	< LOQ	-
EGC	25.68	1.75	1.14	2.46	< LOQ	-
EGCg	78.77	2.85	10.62	4.56	< LOQ	-
EC	6.73	1.60	2.17	3.31	2.51	4.96
ECg	15.18	2.87	7.51	1.40	< LOQ	-
Total Catechins	127.23	-	22.65	-	2.51	-

The dried fresh leaves (green tea) have about five fold more free catechins than the black tea. When the green tea leaves are fermented to form black tea the catechin monomers polymerize to form theaflavins and thearubigens leaving less free catechins in black tea. The catechin content of bottled black tea is between 5 and 10 times less than that of black tea 1% (w/v) liquor. This is based on the content of EC that has the lowest level of detection and thus easily quantified in the bottled black tea. The caffeine content is about half of that found in 1% (w/v) black tea. In the analyzed commercial brand of bottled tea most catechins are below their LOQ's. If we assume that no caffeine was added, these results indicate that the catechins are less stable than the caffeine during the bottling process. Previously it was shown that tea catechins undergo epimerization during heat sterilization (Seto, 1997), (Komatsu, 1991). The extra peaks in the iced tea electropherogram (Fig. 2.16B) could be epimers such as (-)-GCg which may have formed from (-)-EGCg. At pH higher than 6 the catechins dis-

appear due to oxidation and polymerization (browning) (Komatsu, 1991). These polymers may cause the upward baseline drift found in black iced tea samples (Fig. 2.16B).

For green tea analysis a 0.1% (w/v) infusion is sufficient to analyze all the compounds except for theanine and catechin which is below the LOQ. To quantify theanine and catechin, a 0.5% (w/v) infusion is required. At this concentration caffeine, EGCg and EGC exceed the upper limits of linearity, necessitating two electrophoretic runs for quantification of all the compounds. All the compounds in black tea can be quantified from a 0.5% (w/v) infusion in a single run.

For MEKC, with the pseudo-stationary phase, the resolution (R_s) can be expressed as in equation 5.

$$R_s = \frac{\sqrt{N}}{4} \bullet \frac{\alpha - 1}{\alpha} \bullet f(k') \qquad (5)$$

The function $f(k')$ is defined as shown in equation 6. It is logical that the resolution will be the best when $f(k')$ reaches a maximum. The optimum k' is defined as the k' where $f(k')$ is at its maximum The optimum k' for a given set of conditions is calculated according to the migration times for the t_0 and the t_{mc}. Equation 5 (Terabe, 1985) and equation 6 (Wätzig, 1998) can be used for the calculation of the optimum k' for our conditions.

$$f(k') = \left(\frac{k'}{1+k'}\right)\left(\frac{1 - t_0 / t_{mc}}{1 + (t_o / t_{mc})k'}\right) \qquad (6)$$

The dependence of the function $f(k')$ on k' is shown in Figure 2.17 where t_0=5.86 min and t_{mc}=36.81 min. The maximum of $f(k')$ is normally attained at intermediate k' values. Since $f(k')$ is determined by t_0 and t_{mc}, the optimal k' (k'_{opt}) can be defined as

in equation 7. The optimum k' is an indication of the k' region where the peaks should elute to obtain the maximum possible resolution.

$$k'_{opt} = \sqrt{\frac{t_{mc}}{t_0}} \qquad (7)$$

The optimum k' under our optimum conditions is calculated as 2.5 and all the capacity factors of the optimum conditions range between 0.5 and 2.5 except for theanine with a k' of 0.2.

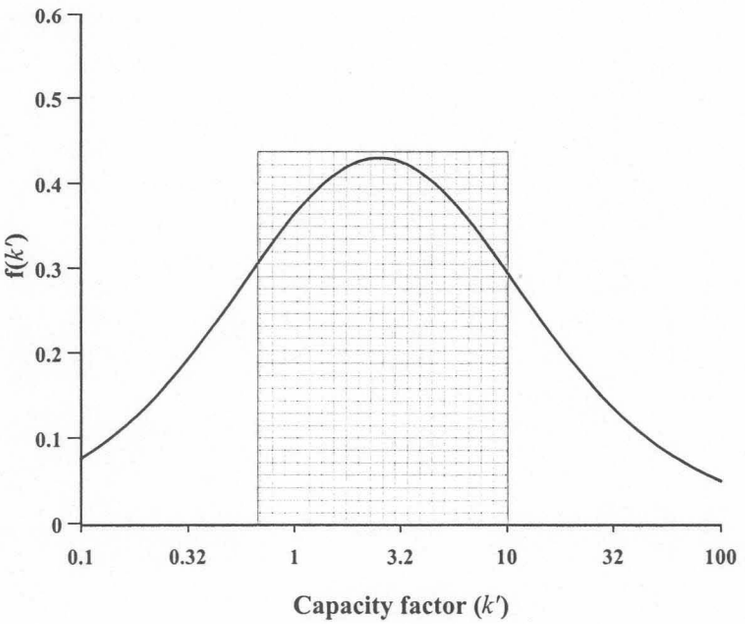

Figure 2.17 The determination of the optimum capacity factor. The region where good separation should be achieved under our conditions is shown with the grid. All the analytes with a k' of 0.5 to 10 can theoretically be separated with a good resolution.

3.9 Optimum condition

We have found that 100 mM SDS, 25 mM phosphate, 6% (v/v) methanol at pH 7.0 provided adequate resolution, consistent migration times and speedy analysis for all tea samples. The best resolution was found at 14 kV. The optimum conditions for

analysis of bottled ice tea samples are 100 mM SDS, 25 mM phosphate, 5% (v/v) methanol at pH 7.0, 14 kV applied voltage on a 50 cm effective length uncoated fused silica capillary column.

4 Conclusion

A new MEKC method has been developed for analyzing tea samples. The method was used for analysis of the catechins, gallic acid, caffeine and theanine content in fresh leaf extracts as well as green and black tea liquors. A slight modification to this method was used to analyze bottled ice tea fortified with ascorbic acid.

The five most abundant tea catechins, theanine, ascorbic acid, caffeine and gallic acid have been separated and quantified with one analysis of approximately 13 min. A few small unidentified peaks have been observed in the green and black tea samples. It is possible that other minor catechins form these unidentified peaks. The equilibration time is 6.5 min extending the time between injections to under 20 minutes. The effect of the chromatographic variables on the resolution and capacity factors presented here may enable other workers to select conditions suitable for their applications. This MEKC method is convenient and precise. Thus it can be used for routine analysis of tea liquors and bottled teas. The separation order is similar to that reported for other MEKC (Horie, 1997), (Horie, 1998 (b)), (Larger, 1998) and reverse phase HPLC methods (Goto, 1996) where the gallated catechins elute later than their ungallated counterparts. This method may possibly be improved in the future to detect other organoleptically important compounds such as aspartic and glutamic acid in tea and dehydro-ascorbic acid in bottled tea. This method was published recently (Aucamp, 2000).