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# Analysis of black tea theaflavins by non-aqueous capillary electrophoresis

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**Abstract**

In this study a new capillary electrophoresis (CE) method was developed to quantify the four major theaflavins occurring in black tea. Where aqueous based CE methods showed poor selectivity and considerable band broadening, non-aqueous CE achieved baseline separation of the theaflavins within 10 min. The effects of the organic solvent composition and background electrolyte concentration on the separation selectivity and electrophoretic mobilities were investigated. Our optimized separation solution consisted of acetonitrile–methanol–acetic acid (71:25:4, v/v) and 90 m*M* ammonium acetate. This method was used to analyze three black tea samples. 

*Keywords:* Theaflavins; Flavanols; Tea; Food analysis; Non-aqueous capillary electrophoresis

## 1. Introduction

The value of tea is determined by its quality as perceived during organoleptic evaluation by tea tasters. Although the professional tea taster has expert knowledge on tea and a fine tuned sensory ability to identify and describe the various attributes of a tea liquor, the scores between different tea tasters may differ according to personal preferences or the demands of the market. Methods for analysing tea samples for predicting their value are required by the tea industry. Such methods can help with the breeding and selection programme where insufficient material is available for scoring by tea tasters. Analytical methods can also be used during manufacturing for process optimization and quality control.

During black tea manufacture, the fresh leaves are macerated to disrupt the cells and sub-cellular compartments. This allows cytoplasmic polyphenol oxidase (PPO) (EC 1.10.3.1) to oxidize the flavan-3-ols in the vacuoles. Although not well understood, tea leaf peroxidase also seems to have an influence on the oxidation of the flavan-3-ols. During the manufacturing process of black tea, the majority of the monomeric flavan-3-ols are oxidized and polymerized to form thearubigins (TRs) and theaflavins (TFs) [1,2]. The diverse group of TRs is still poorly understood without sufficient analysis methods for their characterization. The TFs, on the other hand, are well characterized and show significant correlation with quality. A theaflavin is formed from one trihydroxyflavan-3-ol and one dihydroxyflavan-3-ol. If both the trihydroxy- and dihydroxyflavan-3-ol are gallated, a theaflavin-digallate (TF-dg) is formed. If

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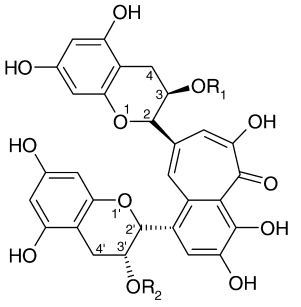
neither substrate is gallated, free theaflavin (TF-f) forms. If the trihydroxyflavan-3-ol is gallated the theaflavin-3-monogallate (TF-A) forms, while if only the dihydroxyflavan-3-ol is gallated, the theaflavin-3′-monogallate (TF-B) is formed [3] (Fig. 1). The flavan-3-ol content of the fresh leaf and the manufacturing procedure determine the TF composition of black tea.

It is important to continuously strive to raise the quality of tea [4]. For Southern African black tea, there is a significant correlation between total TF content and value [5]. However, research in Kenya has indicated that the relative amounts of the individual theaflavins might have a considerable effect on the quality of the black tea [6–8]. Analyzing individual TFs can be used to determine their influence on the quality of Southern African black teas. A fast and sensitive method to monitor the formation of the individual TFs during manufacturing will be of great help in optimizing the manufacturing process.

The method most widely used for measuring TFs is the spectrophotometric method involving flavagnost reagent (2-aminoethyl diphenylborate in ethanol) [9]. The limitation of the flavagnost method is its inability to quantify the individual TF components. Currently the preferred methods for determining individual TFs are based on isocratic or gradient HPLC methods [10,11]. These methods require large amounts of solvents and long analysis times. A method for analyzing TFs using gas chromatography (GC) has been developed [12]. This method however has the disadvantage of requiring derivatization before analysis.




i Free theaflavin (TF-f)
R1 = H
R2 = H

ii Theaflavin-3-gallate (TF-A)
R1 = 3,4,5-trihydroxybenzoyl
R2 = H

iii Theaflavin-3'-gallate (TF-B)
R1 = H
R2 = 3,4,5-trihydroxybenzoyl

iv Theaflavin-3,3'-digallate (TF-dg)
R1 = 3,4,5-trihydroxybenzoyl
R2 = 3,4,5-trihydroxybenzoyl

Fig. 1. Structures of the four major theaflavins analyzed.

A capillary zone electrophoresis (CZE) method was developed to determine both catechins and TFs. Poor sample stability and high relative standard deviation (RSD) values were encountered when analysing TFs with this method [13]. Other researchers also had difficulty in detecting the TFs in their micellar electrokinetic (capillary) chromatography (MEKC) method [14]. Non-aqueous capillary electrophoresis (NACE) is a method that can be used to separate and analyze compounds that are difficult to analyze in aqueous systems. Various researchers have successfully applied NACE for the analysis of natural compounds [15–17], lipophyllic compounds [18], aromatic compounds [19,20] as well as pharmaceuticals, food compounds and compounds occurring in biological fluids [21].

We report here a new NACE method developed specifically for determining the individual TF composition in black tea.

## 2. Conditions

### *2.1. Instrumentation*

Capillary electrophoresis was performed on a HP $^{3D}$CE system using a fused-silica capillary column from Bio-Rad (Scientific Group, JNB, South Africa) with a diameter of 50 μm. For the CZE method a capillary with a total length of 59 cm and an effective separating length of 51 cm was used. Detection was by UV absorbance at 280 nm and wavelength scans were performed from 190 to 500 nm using the diode array detector. For the NACE method the total length of the capillary was 40 cm, giving an effective separating length of 32 cm. The separation was monitored at 380 nm.

The apparent pH values (pH*) of the background electrolyte solutions were measured with a Mettler Toledo MP220 pH Meter with a glass electrode at room temperature. The electrode was calibrated using standard aqueous buffers, pH 4 and 7.

### 2.2. Reagents

Three different commercial black teas were bought at a local supermarket. The TF standards used in calibrating the capillary electrophoresis (CE) for analyzing the TF profiles were kind gifts from Dr. Y. Hara (Mitsui Norin, Japan). Isobutyl methyl ketone (IBMK) complying with ACS standards was purchased from Merck (Merck NT Laboratory Supplies, JNB, South Africa). Acetonitrile (ACN) and methanol (MeOH) used for CE analysis were of HPLC grade and purchased from BDH (Merck NT Laboratory Supplies). Macherey–Nagel MN 617 filter paper was purchased from Separations, JNB, South Africa. All other reagents and solvents were of analytical grade.

### 2.3. Analytical conditions

The running buffer used for the CZE method consisted of 50 m*M* $K_2SO_4$ and 600 m*M* boric acid titrated to pH 7.0 using 1.0 *M* NaOH. The applied voltage was 22.5 kV and the temperature was maintained at 25°C. The sample was injected pneumatically for 5 s (50 mbar). The capillary was rinsed with water for 1 min, 0.1 *M* NaOH for 2 min and then again with water for 2 min after each analysis. At the start of each analysis the capillary was also rinsed with running buffer for 2 min before injection of sample.

For the NACE technique the running solution consisted of ACN–MeOH–acetic acid (71:25:4, v/v) and 90 m*M* ammonium acetate. The analytical conditions for the NACE method was a final applied voltage of 27.5 kV and the temperature was maintained at 18.5°C. Due to current cut-off problems, the voltage was initially increased to 10 kV over the first minute, and then to 27.5 kV over the next 2 min. The sample was injected pneumatically for 5 s (50 mbar). Before each analysis the capillary was rinsed with 1.0 *M* NaOH for 2 min, followed by water for 0.5 min, ACN–MeOH (75:25, v/v) for 0.5 min and finally running solution for 1 min. After each analysis the capillary was rinsed with MeOH for 0.5 min and water for 1 min. This elaborate rinsing schedule was used to ensure no precipitation of salts when changing between non-aqueous and aqueous solvents. At the end of each day the column was rinsed for 20 min with 1.0 *M* NaOH, 5 min with water, 15 min with MeOH and 5 min with water.

### 2.4. Preparation of samples and standard

For the CZE method, dried tea (DT) was extracted by adding 6 g black tea to 250 ml boiling deionized water in a preheated thermos flask and shaking for 10 min. During this time the thermos flask was continuously shaken on a horizontal shaker at ~90 rev./min. The tea infusion was then filtered through Macherey–Nagel MN 617 fluted filter paper. After cooling to room temperature, the tea infusion was extracted with one volume of chloroform and the chloroform fraction discarded. The aqueous tea extract was then extracted with one volume IBMK and the aqueous phase discarded. The IBMK extract was then once again retained after extraction with 5% sodium hydrogen carbonate (w/v). The IBMK (50 ml) was then evaporated in a Buchi Rotavapor-RE 120 and the remaining residue redissolved in 40% (v/v) aqueous ethanol (5 ml). This solution was then stored at −20°C until analysis.

For the NACE method 2 g DT samples were extracted similarly to the CZE method with 100 g boiling water. This tea extract was then filtered through Macherey–Nagel MN 617 fluted filter paper. An aliquot (10 ml) of the filtrate was extracted with one volume (10 ml) of IBMK at room temperature. After phase separation, samples of 1 ml of the upper IBMK phase were transferred to separate 4.5-ml amber vials. The IBMK was removed by evaporation on a 40°C heating block under a stream of nitrogen gas. The vials were flushed with nitrogen gas and sealed. The extracts were stored in the dark at room temperature and analyzed within 48 h. Immediately prior to the analysis on the CE the residue was redissolved in 100 μl loading solution consisting of ACN–MeOH–acetic acid (74.5:25:0.5, v/v). The TF standards were also dissolved in the loading solution for analysis.

### 2.5. Method validation

Reproducibility tests were performed to determine both intra-day and inter-day variation in migration times. The statistical evaluation was carried out on TF standards being analyzed sevenfold for the intra-

day reproducibility test and data from 7 days with five repeats per day for the inter-day reproducibility test. Repetitive runs were carried out with commercial black tea samples to determine the reproducibility of both the extraction and analysis of the samples. Each black tea sample was extracted four separate times and each extract was analyzed in fourfold. The RSD values and the means were determined with the Fig.P (version 2.98) software package [22].

## 3. Results and discussion

### *3.1. Aqueous based methods*

The aim of this study was to develop a CE method for analyzing the individual TFs in black tea infusions. First attempts were made with aqueous buffers using the methods of CZE and MEKC. Of the two aqueous methods CZE was more suitable. We have been unable to analyze TFs by MEKC and others have reported similar problems [14].

The CZE separations of the TFs were based on its complexation with borate [23–26]. The borate concentration and pH had the most significant influence on the separation of the analytes. Although the four TFs could be separated, peak broadening was a significant disadvantage of this method (Fig. 2). High buffer concentration, together with potassium sulfate as an additive, decreased peak broadening. Leading fronts observed were most probably caused by conductivity differences between the zone of analytes (TFs) and the carrier electrolyte (boric acid), as well as interactions with the capillary wall [27].

Another significant drawback of the CZE method was the elaborate extraction procedure of the TFs. The CZE sample preparation procedure had additional chloroform and sodium hydrogen carbonate washing steps, compared to the NACE sample preparation procedure. Without the chloroform washing step capillary blockage occurred, most probably due to the precipitation, or interaction with the capillary wall, of some lipophyllic compounds. The sodium hydrogen carbonate washing step was necessary for additional clean up of the sample to prevent interference of the TF peaks. Peak broadening resulted in unacceptable low TF peak heights at 380 nm. This

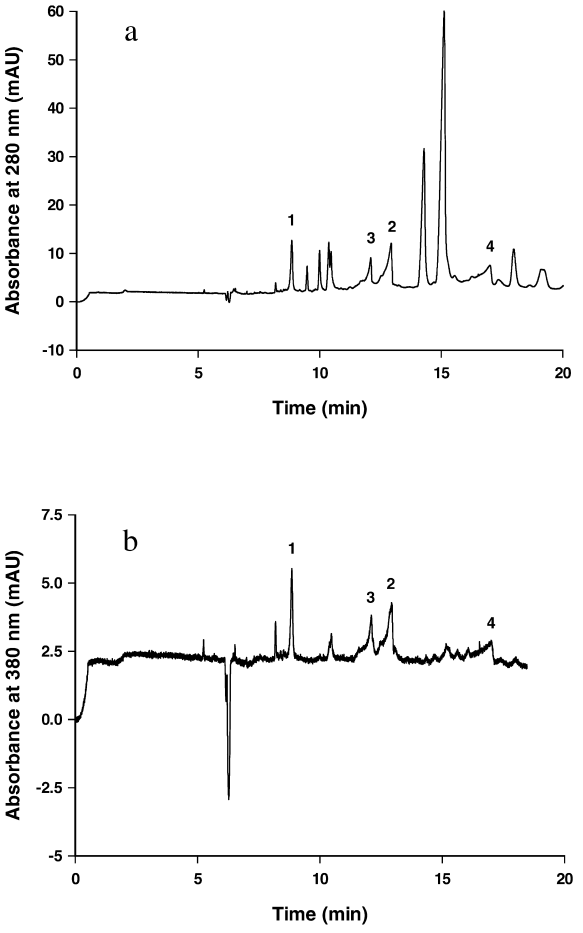



Fig. 2. Electropherogram of a TF extract prepared from a commercial black tea sample. Running buffer 600 m*M* boric acid and 50 m*M* $K_2SO_4$ titrated to pH 7.0 with 1.0 *M* NaOH. Capillary length 59 cm (51 cm to the detector), 50 μm I.D., sample injection 50 mbar for 5 s. Applied voltage 22.5 kV, capillary temperature 25°C and detection at (a) 280 nm and (b) 380 nm. Peaks 1, TF-f; 2, TF-A; 3, TF-B; 4, TF-dg.

method was discontinued because of the low efficiency and high variation in quantification of complex black tea samples.

### *3.2. Factors influencing the NACE analysis*

NACE has emerged as an additional CE method for the analysis of compounds that are difficult to separate in aqueous buffers due to low solubility or lack of selectivity in aqueous media [18,28,29]. Due to the different chemical and physical properties of

organic solvents compared with water, selectivity can be improved. For our analytes the main consideration for using NACE was to decrease the band broadening effect observed in aqueous media and to enhance the separation efficiency.

Factors enhancing band broadening include the injection process, electrophoretic dispersion, Joule heating, wall adsorption, local turbulences due to non-uniformly charged capillary walls, and hydrostatic flow. NACE has the most pronounced effects on Joule heating due to the lower conductivities associated with non-aqueous media. Wall adsorption effects might also be significantly influenced by using organic solutions due to their effect on the solubility and the p$K_a$ of the analytes [30].

Pollutant phenols have been analyzed with a non-aqueous solution consisting of ACN–MeOH–acetic acid, ammonium acetate and potassium hydroxide [28]. Because of the polyphenolic nature of the TFs we decided to develop a non-aqueous method, following a similar strategy.

Acetone was used as the neutral marker in this study when calculating the electrophoretic (actual) mobilities. Because of the gradual increase of the applied voltage over the first minute and then again over the next 2 min at a different rate, the final voltage of 27.5 kV was not used as the applied voltage, but the average voltage as determined at the migration time of each analyte. This slow ramping of the voltage was necessary because current cut-off problems were experienced with fast ramping. Due to current cut-off the final voltage of 27.5 kV was not used for all the running solutions analyzed. For each non-aqueous solution the highest voltage that did not cause current cut-off problems was used. This was in the range of 22 to 30 kV.

The effect of MeOH content on the actual electrophoretic mobilities of the TFs is shown in Fig. 3. The TFs showed a decrease in electrophoretic mobility with an increase in MeOH. The migration of the TFs is, however, also dependent on the extent and direction of the electroosmotic flow (EOF) (Table 1). The apparent mobilities also decreased with an increase in MeOH content. The decreased apparent mobilities at a higher MeOH content can most properly be explained by the decrease in the EOF. This change in EOF is the result of the change in ion mobilities as expected for mixtures of solvents that

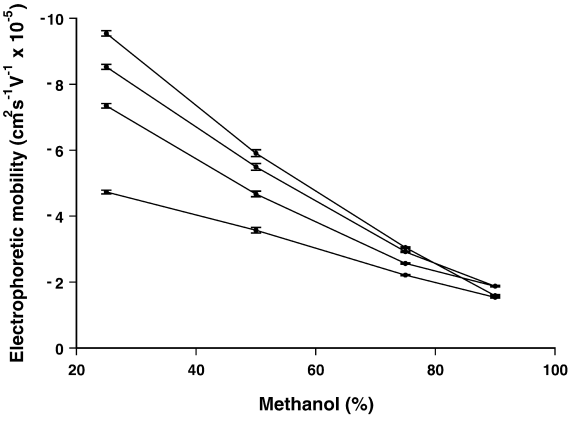



Fig. 3. Electrophoretic mobilities of the four major TFs in different MeOH–ACN compositions containing 90 m*M* ammonium acetate and 4% (v/v) acetic acid. Capillary length 40 cm (32 cm to the detector), 50 μm I.D., sample injection 50 mbar for 5 s. Capillary temperature 18.5°C and detection at 380 nm. The mean and standard deviation of three different runs for each data point is shown.

have different dielectric constants and viscosities, as described by the Von Smoluchowski equation [18]. The increased viscosity and decreased dielectric constant of the media containing higher amounts of MeOH should result in a decreased EOF. ACN has a higher dielectric constant and lower viscosity than MeOH. This tendency was also observed by other researchers [18]. Decreased EOF mobilities were also observed for the additions of organic solvents to an aqueous buffer, with the EOF decreasing more with the addition of MeOH than with the addition of ACN [31].

In solvents with low hydrogen acceptor abilities, such as ACN, heteroconjugated anion formation may play an important role [32]. Anions added to ACN undergo very weak solvation and will thus rather interact with a stronger hydrogen donor Brønsted acid than be solvated. In our application the TFs and other polyphenolic substances in the sample will act as Brønsted acids and undergo ion pairing with the separation electrolyte. At higher ACN content increased ion pairing would decrease the ionic strength, which will lead to an increase in the EOF and the apparent mobilities of the analytes. The actual mobilities of the analytes will also increase due to the ion pairing [18].

The observed decrease in apparent mobilities (and

Table 1
The EOF mobilities and apparent pH for different non-aqueous solutions

| MeOH (%) | Acetic acid (%) | ACN (%) | Ammonium acetate (m*M*) | EOF $cm^2\ min^{-1}\ V^{-1}\times10^{-3}$ | Apparent pH (pH*) |
|---|---|---|---|---|---|
| 10 | 4 | 86 | 90 | | 6.1 |
| 25 | 4 | 71 | 90 | 12.506 | 6.2 |
| 50 | 4 | 46 | 90 | 11.063 | 6.1 |
| 75 | 4 | 21 | 90 | 5.731 | 5.9 |
| 90 | 4 | 6 | 90 | 2.489 | 5.7 |
| 25 | 4 | 71 | 10 | 22.843 | 5.4 |
| 25 | 4 | 71 | 20 | 20.321 | 5.6 |
| 25 | 4 | 71 | 30 | 17.619 | 5.7 |
| 25 | 4 | 71 | 60 | 13.884 | 6.0 |
| 25 | 4 | 71 | 90 | 12.506 | 6.2 |
| 25 | 4 | 71 | 120 | 9.266 | 6.3 |
| 25 | 1 | 71 | 90 | 11.764 | 7.2 |
| 25 | 2 | 71 | 90 | 11.748 | 6.8 |
| 25 | 4 | 71 | 90 | 12.506 | 6.2 |
| 25 | 6 | 71 | 90 | 11.588 | 5.8 |
| 25 | 8 | 71 | 90 | 11.671 | 5.4 |

EOF) at even a lower MeOH content of 25% (except for TF-f) and lower (results not shown) might be due to an effect of the higher ACN content on the effective charge to solvation radius ratio of the analyte. This tendency was also observed for the separation for pharmaceuticals in non-aqueous media [33]. The reduced EOF at high ACN content might also be because of a decrease in the effective charge at the capillary surface due to an increase of the $pK_a$ of silanol groups, which would decrease the $\zeta$ potential at the silica surface.

The most important effect on the selectivity of organic solvents on the analytes is due to its influence on the $pK_a$ values of the analytes. The effect of MeOH [34] and of ACN [35] on the $pK_a$ values are described based on the theoretical model of the transfer activity coefficient (medium effect). For the addition of the above-mentioned solvents to water, an increase in the $pK_a$ values was observed. An increase in the $pK_a$ values of the TFs might also be responsible for the reduced wall adsorption in the non-aqueous solution system. A more protonated state of the TFs should result in reduced ionic interaction with the negatively charged capillary wall. This also coincides with the ion pairing effect described earlier.

The effect of added acetic acid on the electrophoretic mobilities is shown in Fig. 4. An increase of acetic acid resulted in a decrease in actual mobilities for the TFs. Acetic acid was added to act as a hydrogen-bond donor to overcome possible solubility problems of our non-aqueous solution which consisted of a high percentage of ACN in MeOH. However, the addition of the acetic acid also had a marked effect on the mobilities of the TFs, therefore the amounts of acetic acid added were also opti-

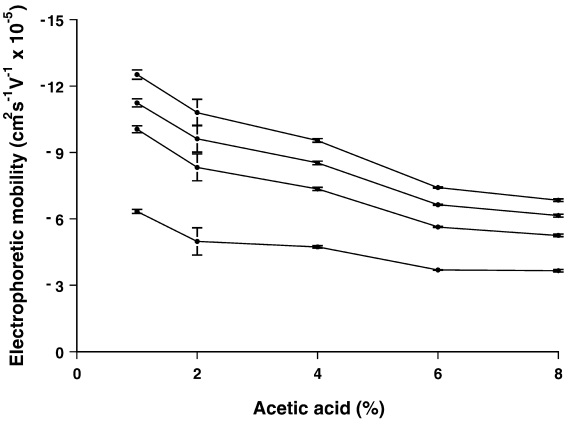



Fig. 4. Electrophoretic mobilities of four TFs in running solutions containing different acetic acid–ACN compositions and 90 m*M* ammonium acetate and 25% (v/v) MeOH. Other experimental conditions as in Fig. 3.

mized. The addition of the acetic acid did not have a considerable influence on the selectivity of the TFs. As seen in Table 1, the addition of acetic acid had a large effect on the pH*, compared to changes of pH* with variations in the MeOH or ammonium acetate content. A decrease in the pH* did however not have the expected decreasing effect on the EOF (Table 1). The decreased mobilities might be due to a decrease in the pH* which should reduce the charge of the anionic TFs, this will then increase the apparent mobility. The increased mobility can also be explained by the ion pairing effect. With the addition of acetic acid as hydrogen donor to ACN, the solubility of ions increases and this will result in reduced ion pairing with the consequent decrease in mobility of the solutes.

Studies in aqueous buffers, predicted a decrease in the solute mobilities and the EOF with an increase in the ammonium acetate concentration due to the increased ionic strength [36]. Although the EOF decreased with increasing ammonium acetate concentration, the TF mobilities increased (Fig. 5). The increase in the mobilities of the TFs and the decrease of the EOF coincided with increased resolution. This observation can be explained by the ion pairing effect. With higher concentrations of ions in the non-aqueous solution, increased ion pairing will result in increased mobilities of the TFs due to the increased charge of the analytes.

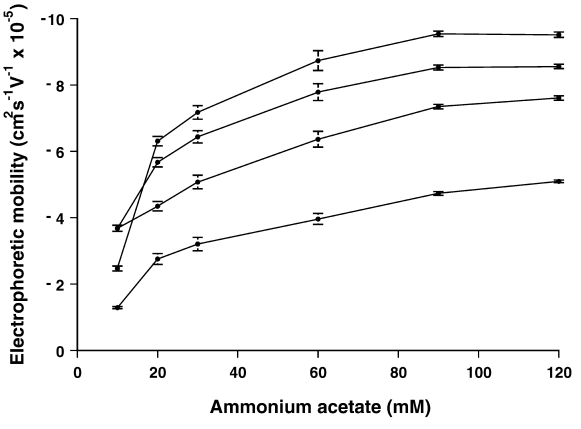



Fig. 5. Electrophoretic mobilities of four TFs in running solutions containing different amounts of ammonium acetate and ACN–MeOH–acetic acid (71:25:4, v/v). Other experimental conditions as in Fig. 3.

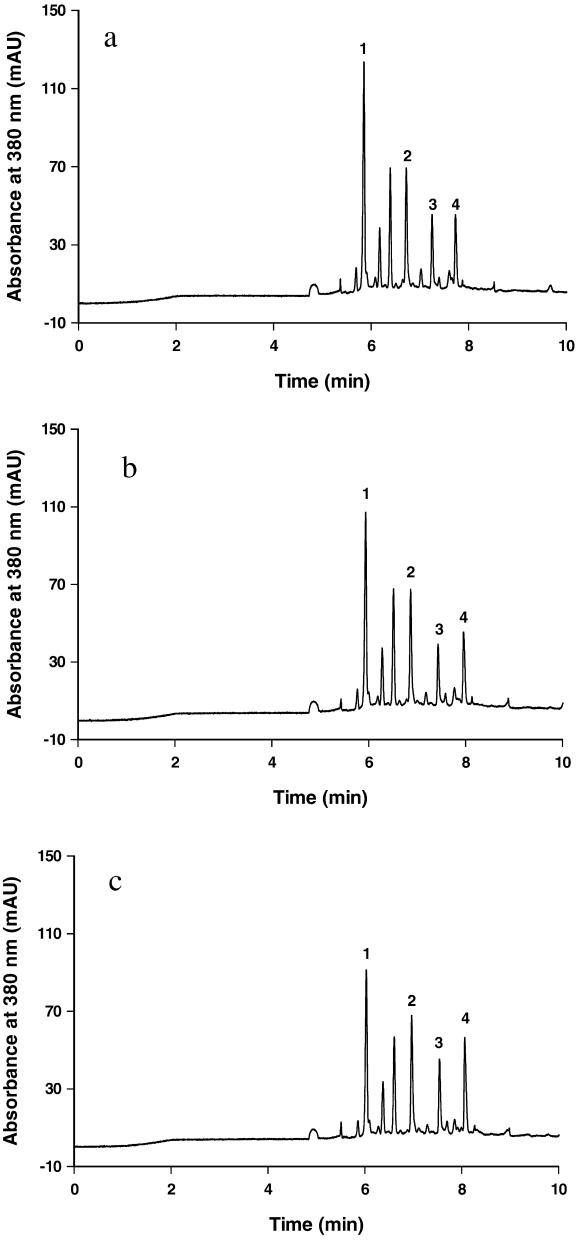



Fig. 6. Electropherogram of a TF extract prepared from commercial black tea samples, (a) Black tea A, (b) Black tea B and (c) Black tea C. Running solution ACN–MeOH–acetic acid (71:25:4, v/v), 90 m*M* ammonium acetate. Applied voltage 27.5 kV. Other experimental conditions as in Fig. 3. Peak identification as in Fig. 2.

Table 2
The variation in individual TF content of three different local black tea samples

| Sample | TF-f | | TF-A | | TF-B | | TF-dg | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean (μmol/g DT) | RSD (%) | Mean (μmol/g DT) | RSD (%) | Mean (μmol/g DT) | RSD (%) | Mean (μmol/g DT) | RSD (%) | Mean (μmol/g DT) | RSD (%) |
| Higher priced | | | | | | | | | | |
| Black tea A | 6.809 | 3.9 | 2.654 | 4.1 | 1.068 | 5.6 | 1.060 | 5.1 | 11.593 | 4.1 |
| Black tea B | 6.073 | 3.5 | 2.661 | 3.8 | 0.943 | 3.8 | 1.118 | 3.3 | 10.793 | 3.4 |
| Lower priced | | | | | | | | | | |
| Black tea C | 4.716 | 4.4 | 2.458 | 5.3 | 1.027 | 5.6 | 1.440 | 5.8 | 9.642 | 4.9 |

The longer migration times and excessive Joule heating are however a disadvantage when using higher concentrations of the background electrolyte. In our method we used the increased separation selectivity observed with an increase in the ammonium acetate concentration, together with the decreased migration times observed with an increase in acetic acid content, to optimize our method. We used a concentration of 90 m*M* ammonium acetate to achieve the desirable resolution, and a relatively high content of 4% acetic acid to decrease the migration times of the four TFs to well under 10 min. Our optimized separation solution thus consisted of ACN–MeOH–acetic acid (71:25:4, v/v) and 90 m*M* ammonium acetate.

### *3.3. Analytical procedure*

The four major TFs elute within 10 min. Column purge and re-equilibration extend the total analysis time by 6 min. The analysis time for TFs on HPLC is significantly longer. Using a simple IBMK extraction of a black tea infusion, the individual TFs could be identified and quantified as shown in Fig. 6. These peaks were identified by their migration times as well as their UV–VIS spectra.

Calibration curves for TF, TF-A, TF-B and TF-dg were linear in the range of interest (100 to 1000 μg/ml), with $r$ values of 0.9998, 0.9992, 0.9995 and 0.9987, respectively. The repeatability ($n=4$) of the method was also evaluated as shown in Table 2. The RSD values for the determination of the individual TF concentrations were smaller than 6% for all the different black tea samples. The variation in the migration times of the TF standards is shown in Table 3. Intra-day variations (1.7%) and inter-day variations were less than 3.6%. The limit of detection (LOD) values were taken as 3 $S/N$ at 380 nm and were 24, 25, 21 and 23 μg/ml for TF-f, TF-A, TF-B and TF-dg, respectively.

The individual TF concentrations of three black tea samples were determined (Table 2). The samples consisted of two higher priced commercial black teas (Black tea A and Black tea B) of above average quality, and one lower priced commercial black tea (Black tea C). True to findings of previous investigations the total TF concentration determined by the NACE method was higher for the higher priced black teas (11.593 and 10.793 μmol/g DT) than for the lower priced black tea (9.642 μmol/g DT). The relative compositions of the individual TFs also differed between the three tea samples. The higher

Table 3
The intra- and inter-day variation of the migration times

| Compound | Intra-day variation ($n=7$) | | Inter-day variation ($n=7$) | |
|---|---|---|---|---|
| | Mean (min) | RSD (%) | Mean (min) | RSD (%) |
| TF-f | 6.05 | 1.4 | 5.90 | 2.6 |
| TF-A | 7.02 | 1.5 | 6.82 | 3.1 |
| TF-B | 7.61 | 1.6 | 7.36 | 3.3 |
| TF-dg | 8.20 | 1.6 | 7.91 | 3.5 |

priced black teas had a higher amount of TF-f and a lower amount of TF-dg. A more thorough investigation into the correlation between the individual TF content and tea quality as perceived by the tea taster can now be undertaken for Southern African teas.

## 4. Conclusion

To our knowledge, no prior method for analyzing tea by NACE existed. Although TFs were already separated by CE [13], this is the first CE method developed for TF analysis only. This NACE method has the advantages of speed, low capillary and running costs compared to the HPLC methods. This new CE method could be used for evaluating new tea clones and optimizing quality during black tea manufacturing.

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