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# Tetraethylammonium tetrafluoroborate: a novel electrolyte with a unique role in the capillary electrophoretic separation of polyphenols found in grape seed extracts

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A capillary electrophoretic method for resolving phenolic compounds found in grape seed extracts was developed using a quaternary ammonium salt as the main electrolyte solution. Seven polyphenols were separated and identified. The separation mechanism seems to involve association between quaternary ammonium cations and the polyphenols. Migration times roughly correlate with the size of the known phenols. The role of the alkyl substituents on the quaternary ammonium ions was investigated and is discussed. The method of analysis proposed herein exhibits high reproducibility in terms of migration times for a complex mixture using a single component background electrolyte.

## 1. Introduction

Increasingly, consumers are being offered and asking for various types of extracts as dietary supplements or 'nutraceuticals'. These are becoming a common part of human diets either by choice or by necessity, as their nutritional value and potential health benefits are being reported by researchers. For instance, consumption of green tea and moderate consumption of red wine has been associated with a variety of health benefits in humans[1] including anticarcinogenic,[2] anticariogenic,[3] and hepato-protective activities.[4]

It is thought that naturally occurring antioxidants, in the form of polyphenols, may be responsible for some of this therapeutic effect. The disease preventing activity arises from the ability of polyphenols to protect low density lipoproteins against oxidation. In general, polyphenols are present as complex mixtures of homologues and isomers with differing degrees and sites of polymerization. The phenolic polymers are mainly composed of monomeric flavan-3-ol units such as the diastereoisomeric pair (+)-catechin and/or (−)-epicatechin units.[5] Depending on the type and the number of units, they form specific oligomeric procyanidins (short-chained polymers). The flavan-3-ol units are linked principally through the 4 and the 8 positions.

Polyphenols contribute to a wide variety of sensory characteristics of wines and teas such as taste and flavor which play an important role in the industrial beverage production.[6] Special consideration in terms of concentration and type of phenolic compounds needs to be taken into account when producing certain types of beverages. Quality assurance and control of these biologically-derived materials can be accomplished through separation and subsequent characterization.

Currently, there is a considerable interest in the analysis of phenolic mixtures from sources such as grape seeds and green teas. Most of the methods employ high-performance liquid chromatography (HPLC),[7–9] and to a lesser extent, gas chromatography.[10] More recently, capillary electrophoresis (CE) has been used for the analysis of polyphenols.[11–14] Capillary electrophoresis is a simple technique with tremendous resolving power, separating individual components of complex mixtures roughly on the basis of charge-to-mass ratios.[15] In comparison with HPLC, CE generally provides better peak shapes and faster analysis. However, CE is often plagued with reproducibility problems.

In CE, alkylammonium salts have been exclusively used as electro-osmotic flow modifiers. The direction of electro-osmotic flow (EOF) may be reversed by simply adding a quaternary alkylammonium salt to the buffer.[16] The main advantage of reversing the EOF has been realized in the analysis of anions.[17] The use of this methodology causes anions to elute faster resulting in shorter analysis times.

Some of the tetraalkylammonium salts reported as flow modifiers include the surfactants cetyltrimethylammonium bromide and chloride and tetradecyltrimethylammonium bromide in which one of the hydrocarbon substituents on the nitrogen is a long-chain hydrocarbon. Presumably, in these systems, the anodic EOF emanates from the tetraalkylammonium groups on the surface of a bilayer of surfactant molecules on the capillary walls in which the hydrocarbon tails of the secondary surfactant layer intercalate with the hydrocarbon tails of the initial surfactant layer.

In electrochemistry, tetraethylammonium tetrafluoroborate (TEA-TFB) has been used extensively as a background electrolyte, because it is very stable, safe to use and provides favorable electrolytic conductivity.[18] Because TEA-TFB generates relatively low currents in CE, Joule heating should not be a problem. The high solubility of this flow modifier allows for its use over a wide range of working concentrations (up to 300 mM).

The present work describes a simple and reproducible electrophoretic method for the determination of phenolic compounds found in grape seed extracts. The method primarily involves the use of a tetraalkylammonium salt in the running electrolyte solution.

## 2. Experimental

### 2.1. Chemicals

(−)-Catechin, (−)-epicatechin, (−)-catechin gallate, (−)-gallocatechin gallate, (−)-epicatechin gallate, gallic acid, resvera-

trol, (−)-gallocatechin, (−)-epigallocatechin, (−)-epigallocatechin gallate, quercetin, rutin, kaemferol, myricetin, caffeic acid, ferulic acid, *p*-coumaric acid, vanillic acid and chlorogenic acid were purchased from Sigma (St. Louis, MO, USA). Gallangin, ellagic acid dihydrate, syringic acid and tetramethylammonium tetrafluoroborate were purchased from Aldrich (Milwaukee, WI, USA). Sinapic acid, 3,4-dihydroxybenzoic acid, tetraethylammonium tetrafluoroborate and tetrapropylammonium tetrafluoroborate were purchased from Fluka (Ronkondoma, NY, USA). Sodium tetraborate pentahydrate, citric acid monohydrate, sodium citrate, sodium hydroxide and hydrochloric acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

### 2.2. Apparatus

All CE experiments were carried out using either a Bio-Rad BioFocus 2000 or a Bio-Rad BioFocus 3000 automated capillary electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) equipped with a UV detector and interfaced with a personal computer. The bare fused-silica capillary (50 μm internal diameter) had a total length of 60 cm (55.4 cm to the detector). Three millimeters of the polyimide coating were burned off for UV detection. The capillaries were obtained from Bio-Rad.

The conditioning of the capillary was done by purging with 0.5 M sodium hydroxide for 10 min, distilled, de-ionized water for 3 min, followed by running buffer for 10 min. Daily, at the beginning of each experimental session, the capillary was rinsed and washed following the above treatment. The washing and rinsing treatment was not required between runs. The best separation was obtained using 20 kV, at a constant temperature of 20 °C. Detection was achieved at 200 nm. Injection was performed hydrodynamically at 5 psi for 0.4 s.

The operating polarity of the CE instrument during the analysis of the grape seed extract depended on the background electrolyte conditions and is detailed in the figure legends.

### 2.3. Grape seed extract sample preparation

The extracts used in the present study were provided by Givaudan Roure Flavors Corporation (Cincinnati, OH, USA). A portion (0.03 g) of the grape seed extract was weighed into a 15 mL sample vial. Water was then added to bring the total weight to 15 g giving a final concentration of 2 mg $mL^{-1}$. Before injection, a 200 μL aliquot of the sample was diluted to a total volume of 1200 μL with the electrolyte solution to bring the concentration to 0.333 mg $mL^{-1}$. Sample and run buffers were de-gassed and filtered using a 0.2 μm filter.

### 2.4. Standards and buffer preparation

The working standard solutions were prepared daily by dissolving the standards in distilled, de-ionized water, and further diluted using the running electrolyte solution. Sodium tetraborate pentahydrate was used to prepare the initial run buffer solutions, and the pH was adjusted using either 0.1 M NaOH or 0.1 M HCl. Borate solutions in the range 20–400 mM and in the pH range 7–11 were prepared. Buffer systems of citric acid–sodium citrate (containing 150 mM tetraethylammonium tetrafluoroborate) were also prepared with citrate concentrations of 10, 20, 50 and 150 mM, (pH of 3.5 and 5). In addition, tetraethylammonium tetrafluoroborate solutions in the range 50–300 mM (pH of ~4) were prepared. Solutions of the other two tetraalkylammonium tetrafluoroborates were prepared only at 50 mM concentration.

## 3. Results and discussion

### 3.1. Sodium tetraborate buffer

Based on previous studies,[19] sodium tetraborate was initially used as the run buffer for the CE separation of phenolic compounds. Borate buffer systems have been used to separate a variety of hydroxyl-containing compounds such as catecholamines,[20] glycosaminoglycans,[21] corticosteroids,[22] carbohydrates[23,24] and pyridylglycamines.[25] Specific complexation between borate ion and certain analytes containing hydroxyl groups resulted in negatively charged borate complexes. The formation of such negative chelates played an important role in the electrophoretic separation of such analytes. Indeed, Palmer and co-workers[22] identified the borate ion as the critical buffer component in the separation of some corticosteroids. It was anticipated that the borate buffer, under basic experimental conditions in which the polyphenols are negatively charged, could effect the separation either based on charge-to-mass ratios of the deprotonated polyphenols or through borate–phenol association.

Fig. 1 shows the separation of polyphenols in the grape seed extract using sodium tetraborate. The electrophoretic conditions shown in the figure were found to be optimum for this particular buffer. Under these conditions, anions (negatively charged polyphenols) appear after the neutral marker. It can be seen from the electropherogram that a fairly good separation was obtained for the first eluting compounds, but not for the later ones. In CE, as well as other separation techniques, polymeric materials commonly exhibit a broad envelope rather than discrete peaks. Hence, the rising baseline could indicate the presence of polymeric phenolic compounds (mainly, polymeric gallic acid esters).[19,26] Use of the sodium tetraborate buffer allowed for the identification of only two polyphenols (epicatechin and catechin) which are labeled in the electropherogram in Fig. 1. Additional peaks in the electropherogram are likely unresolved polyphenols.

Efforts to improve the separation of polyphenols using the borate buffer included changes in the concentration and pH of the electrolyte solution as well as changes in instrumental conditions such as temperature and voltage. However, changing such parameters was ineffective and efforts were shifted towards the use of organic modifiers. Although the addition of modifiers (such as surfactants) to the buffer system seemed to improve the separation, reproducibility in terms of the separation and migration times was difficult to achieve (results not shown).

### 3.2. Citrate buffer with tetraethylammonium tetrafluoroborate

A sodium citrate–citric acid buffer system containing a constant concentration of the salt tetraethylammonium tetrafluoroborate was also evaluated. However, at citrate concentrations higher than 20 mM (50 and 150 mM), baseline stability was difficult to

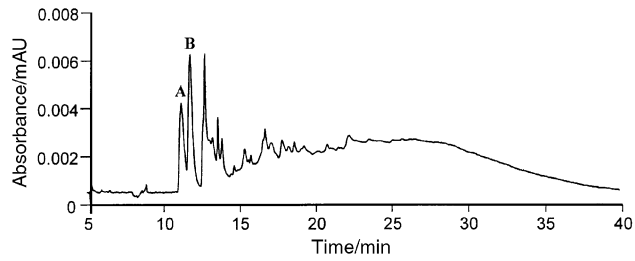



**Fig. 1** Separation of polyphenols using sodium tetraborate (150 mM, pH 8.9). A: Epicatechin. B: Catechin.Voltage: 20 kV, cathodic detection at 200 nm.

maintain and no separation was obtained. Indeed, in the absence of citrate buffer, the migration time reproducibility was better than that at citrate concentrations of 10 and 20 mM. Therefore, further study focused on the evaluation of running systems containing exclusively tetraalkylammonium salts.

### 3.3. Tetraalkylammonium tetrafluoroborate solutions without a buffer

When using the tetraethylammonium tetrafluoroborate as the single component in the background electrolyte, the solution was somewhat acidic (pH ~ 4). Under these conditions, the polyphenols should be uncharged ($pK_a$ ~ 9.5–10.5 for polyphenols).[19] However, the separation of the polyphenols required reversed polarity (*e.g.*, anodic detection) and the migration time of nitromethane was shorter than those of the polyphenols. The delay in the migration of the polyphenols may be attributed to two factors. As illustrated in Fig. 2, polyphenols may associate with the positively charged tetraalkylammonium groups coating the capillary wall. Alternatively, the polyphenols may associate with the tetraalkylammonium ions in the bulk solution. This association could be partially driven by hydrophobic interactions between the polyphenols and the hydrocarbon moieties of the tetraethylammonium ions.

To explore this issue, a series of three tetraalkylammonium tetrafluoroborate salts (tetramethyl-, tetraethyl- and tetrapropyl-) were investigated as the main electrolyte solution for the CE separation of polyphenols. Given the limited solubility of the tetrapropylammonium salt, a concentration of 50 mM for all three was used. Fig. 3 displays electropherograms of the grape seed extract obtained using the different tetraalkylammonium salts. Under these conditions, the EOF was directed towards the anode. From Fig. 3, it can be seen that the magnitude of the EOF as well as the separation of polyphenols was clearly affected by the alkyl chain length of the quaternary ammonium salt. The magnitude of the EOF, as determined from the migration time of nitromethane, increased with increasing alkyl chain length. This may imply that the quaternary alkylammonium cations are being adsorbed onto the fused capillary wall to different extents.

Another aspect that can be noted from Fig. 3 is that the polyphenols migrate after the neutral marker. With respect to the separation, the tetramethylammonium salt did not afford substantial resolution between the individual peaks; resolution was improved by using longer alkyl chains (tetraethyl- and tetrapropyl-). The delayed migration of the polyphenols coupled with the effect of the alkyl chain length on the separation implies that the tetraalkylammonium ion is an active participant in the overall separation mechanism.

Although short analysis times and enhanced peak sharpness were obtained with tetrapropylammonium tetrafluoroborate (see Fig. 3C), there is significant overlap of the early eluting peaks. While higher concentrations of the tetrapropylammonium salt might be expected to increase the separation window, its limited solubility imposes some constraints on this approach. Therefore, further study focused on the use of tetraethylammonium tetrafluoroborate (TEA-TFB) salt solutions.

**3.3.1 Separation mechanism using tetraethylammonium tetrafluoroborate.** For a better understanding of the mechanism of separation using the TEA-TFB salt, the relationship between the migration time (EOF measurement) of the neutral marker and the concentration of the electrolyte solution was studied. From Fig. 4, it can be seen that increasing the concentration of TEA-TFB from 50 to 200 mM caused increased EOF. However, between 200 and 300 mM, the EOF levelled off. Because the EOF is thought to emanate from the tetraalkylammonium coating on the wall, saturation of the capillary walls with tetraethylammonium cation seems to be occurring above ~200 mM.

To distinguish between the contribution of association of the polyphenols with either the tetraethylammonium cations coating the capillary wall or with the free cations in the bulk solution, $\mu_{eff}$,

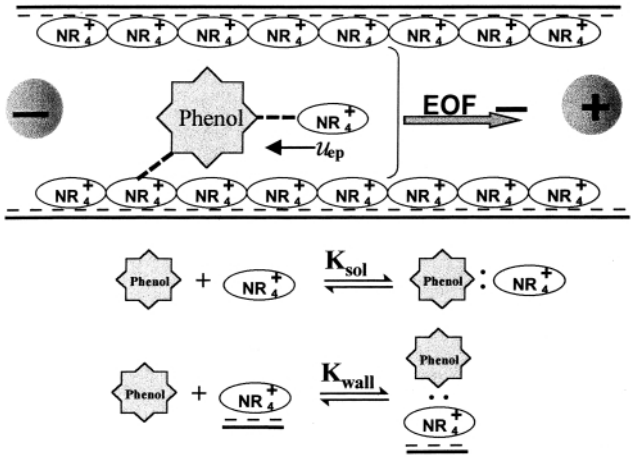



**Fig. 2** Mechanism of separation using tetraalkylammonium tetrafluoroborates. $K_{sol}$: Equilibrium constant for the association between a phenolic compound and a tetraalkylammonium cation in the bulk solution; $K_{wall}$: equilibrium constant for the association between a phenolic compound and a tetraalkylammonium cation immobilized on the capillary wall.

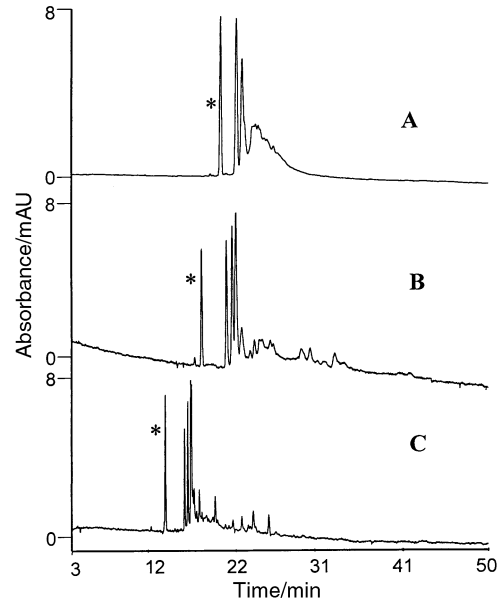



**Fig. 3** Separation of polyphenols using three different tetraalkylammonium tetrafluoroborate salts (50 mM). Voltage: 20 kV with anodic detection at 200 nm. A: Tetramethyl-; B: tetraethyl-; C: tetrapropylammonium salt. * Nitromethane.

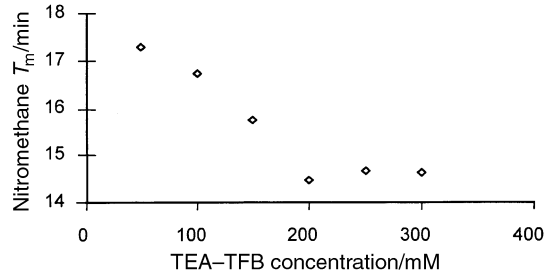



**Fig. 4** Effect of increasing tetraethylammonium tetrafluoroborate concentration on EOF. Voltage: 20 kV.

$$\mu_{eff} = \mu_{meas} - \mu_{eof}$$

the effective electrophoretic mobility of polyphenols, was plotted *versus* the electrolyte concentration (Fig. 5). As illustrated in Fig. 5, a continuous increase in the mobility of polyphenols is observed as the concentration of TEA-TFB increases. The continued increase of the effective electrophoretic mobility of polyphenols with increasing TEA-TFB beyond ~200 mM TEA-TFB (*e.g.*, where EOF levels off) suggests that while both association with the wall-adsorbed and free tetraethylammonium may be important, association with the free tetraalkylammonium ion seems to dominate.

**3.3.2 Characterization of polyphenols using TEA-TFB.** A series of electropherograms of grape seed extracts and a mixture of standards are shown in Fig. 6. Seven polyphenols detected in the grape seed extract and labeled in the electropherograms are listed in Table 1. The other 17 polyphenols mentioned under Experimental were also investigated but were not detected in the extract. The polyphenols detected were identified by spiking the grape seed extract sample with phenolic standards, and also by comparing their migration times with the migration time of the standards. In general, of the seven polyphenols identified, low molecular weight species emerged earlier than those having high molecular weights.

One major benefit obtained by coating the capillary wall with quaternary ammonium ions is that their charge is independent of pH. Most reproducibility issues in CE seem to be wall-related, presumably because of the role that the surface silanols play in the EOF. The electropherograms shown in Fig. 6 were obtained under optimized separation conditions on two different CE instruments and on three different days. Electropherograms A–C were obtained on one instrument and the other two electropherograms (D and E) were obtained on a separate instrument using a different column. As can be seen from Fig. 6, a highly reproducible separation of the polyphenols was obtained. Table 2 lists the average migration times and reproducibilities (standard deviation) of the migration times of the neutral marker nitromethane and all seven phenolic compounds. The average migration times were the means of four inter-day replicates. The standard deviations of the

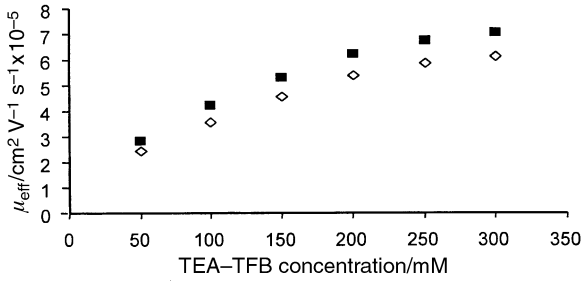



**Fig. 5** Effective electrophoretic mobility of two components of the grape seed extract as a function of tetraethylammonium tetrafluoroborate concentration; epicatechin (◇), catechin (■).

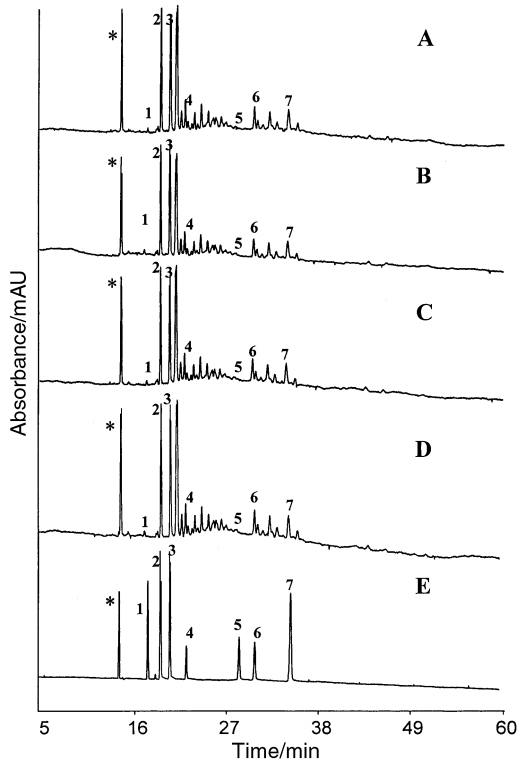



**Fig. 6** Separation of polyphenols using tetraethylammonium tetrafluoroborate (150 mM, pH 4). Voltage: 20 kV with anodic detection at 200 nm. A–D: Grape seed extract. E: Polyphenol standards. * Nitromethane.

**Table 1** Polyphenols detected

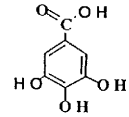
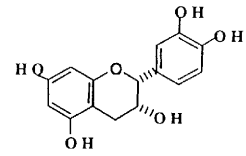
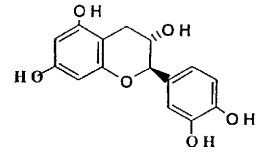
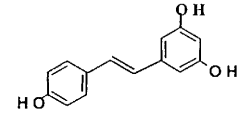
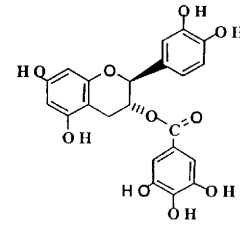
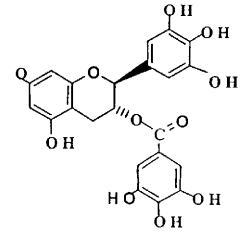
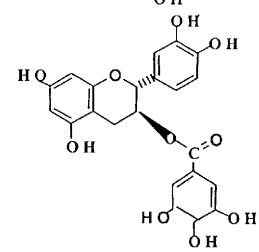
| Name | Molecular formula | Structure |
|---|---|---|
| **1** Gallic acid | $C_7H_6O_5$ |  |
| **2** (−)-Epicatechin | $C_{15}H_{14}O_6$ |  |
| **3** (+)-Catechin | $C_{15}H_{14}O_6$ |  |
| **4** Resveratrol | $C_{14}H_{12}O_3$ |  |
| **5** (−)-Catechin gallate | $C_{22}H_{18}O_{10}$ |  |
| **6** (−)-Gallocatechin gallate | $C_{22}H_{18}O_{11}$ |  |
| **7** (−)-Epicatechin gallate | $C_{22}H_{18}O_{10}$ |  |

**Table 2** Reproducibility of migration times

| Chemical compound | Migration time/min ($n$ = 4) |
|---|---|
| Nitromethane | 14.67 ± 0.09 |
| Gallic acid | 17.64 ± 0.17 |
| Epicatechin | 19.44 ± 0.15 |
| Catechin | 20.55 ± 0.16 |
| Resveratrol | 22.33 ± 0.18 |
| Catechin gallate | 28.34 ± 0.26 |
| Gallocatechin gallate | 30.52 ± 0.22 |
| Epicatechin gallate | 34.57 ± 0.24 |

migration times were less than 0.24 min (RSD < 0.95%) under the optimized separation conditions.

## 4. Conclusions

Successful separation and tentative identification of some phenolic constituents of grape seed extracts has been achieved using tetraethylammonium tetrafluoroborate as the main electrolyte solution. In this system, the use of a buffer system was unnecessary. The electrophoretic method proved to be simple and reliable, providing good peak resolution and excellent reproducibility in terms of migration times. The excellent reproducibility may be the result of the tetraethylammonium cations coating the capillary walls with a permanent charge group that is not subject to pH-induced variations in ionization. Also, in this particular study, the size of the uncharged polyphenols and their different degrees of association with the tetraalkylammonium ions seemed to provide effective electrophoretic mobility differences for successful and effective separations.

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