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ARTICLE

Determination of Total Antioxidant Capacity of Commercial Beverage Samples by Capillary Electrophoresis via Inline Reaction with 2,6-Dichlorophenolindophenol

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This paper demonstrates proof-of-concept for the use of electrophoretically mediated microanalysis (EMMA) as a new approach to the determination of total antioxidant capacity (TAC). EMMA is a low-volume, high-efficiency capillary electrophoretic technique that has to date been underutilized for small molecule reactions. Here, nanoliter volumes of 2,6-dichlorophenolindophenol (DCIP) reagent solution are mixed with an antioxidant-containing sample within the confines of a narrow-bore capillary tube. The mixing is accomplished by exploiting differential migration rates of the reagents when a voltage field is applied across the length of the capillary tube. The ensuing electron transfer reaction between DCIP and the antioxidant(s) is then used as a quantitative measure of the TAC of the sample. Linear calibration using either redox form of DCIP is accomplished with standard solutions of ascorbic acid. Several commercial beverage samples are analyzed, and the TAC values obtained with the reported methodology are compared to results obtained with the widely used ferric reducing antioxidant power (FRAP) spectroscopic method. For the analysis of real samples of unknown ionic strength, the method of standard additions is shown to be superior to the use of external calibration. This easily automated EMMA method may represent a useful new approach to TAC determination.

KEYWORDS: Antioxidant capacity; TAC; electrophoretically mediated; EMMA; capillary electrophoresis; 2,6-dichlorophenolindophenol; DCIP

INTRODUCTION

Antioxidants help prevent oxidative damage to biomolecules caused by free radicals and are thought to aid in the prevention of many health problems, such as heart disease, cancer, and inflammatory diseases (*1*). Thus, the measurement of antioxidants in consumer products is of interest. Separations-based analytical approaches to identifying and quantifying the individual antioxidants found in a food or beverage sample are challenging, but a few approaches have been reported (*2*−*5*). Due to the chemical diversity of antioxidants, and the complex mixtures of antioxidants often found in many real samples, the successful use of any single separation technique has proven to be difficult. In addition, the collective effect of a complex mixture of antioxidants is often different from the sum of the contributions of the individual antioxidants in the mixture (*6*). These factors, along with commercial pressure for a simple numerical representation of the antioxidant content in a sample, have led to the useful concept of total antioxidant capacity (TAC). Instead of being concerned with the individual responses of each antioxidant, TAC considers the aggregate strength of all antioxidant compounds present in a given sample (*7*−*14*).

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There have been numerous laboratory methods for TAC determination reported in the literature (*8*−*15*), and these methods vary widely in both the chemical approach and the resulting numerical values obtained for TAC. The chemistry behind several of these methods was the topic of a very nice recent review (*15*). The chemistry employed in TAC analyses generally falls into two categories: methods involving electron transfer (ET) reactions and methods involving hydrogen atom transfer (HAT) reactions. Regardless, the analyses generally involve mixing a sample solution with one or more standard reagent solutions while changes in either absorbance or fluorescence are monitored. The most widely used methods are the ferric reducing antioxidant power (FRAP) assay (*8*), the Trolox equivalent antioxidant capacity assay (TEAC) (*9*), and the oxygen radical absorbance capacity (ORAC) assay (*10*). Although each of these methods is designed to measure the aggregate antioxidant capability of a given sample, the methods are performed under widely differing conditions, so it is perhaps not surprising that these methods often produce differing numerical results (*11*−*14*), making attempts to quantitatively compare data obtained with different procedures ill-advised. Nonetheless, TAC has become an increasingly popular metric by which antioxidant properties can be described. Indeed, TAC values are now found on the labels of many commercial beverages in the United States.

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Within the past two decades, capillary electrophoresis (CE) has become a viable analytical technique (*16*−*21*). CE gained a great deal of credibility as the technique that allowed the human genome to be solved several years ahead of schedule (*17*). The general advantages of CE-based analyses include low sample volume requirements (and correspondingly low amounts of chemical waste), rapid analysis times, and high efficiency. These compelling aspects of CE-based methods, in capillary or microfluidic chip format, position CE as an increasingly attractive alternative to traditional methods of analysis. Here we consider the utility of electrophoretically mediated microanalysis (EMMA), a CE-based approach for performing low-volume inline chemical reaction(s) to determine TAC. EMMA, which was first reported by Bao and Regnier in 1992 (*22*), is an excellent analytical tool for inline mixing of nanoliter volumes of reagent(s) and a sample for chemical analysis. EMMA is performed in a fused silica capillary, and the mixing of chemical reagents within the capillary is accomplished by applying a voltage field, exploiting the differences in the electrophoretic mobilities of the reactants. The EMMA technique retains all of the attractive features of CE methods, such as small sample volumes, minimal waste, use of a single online flow-through detector, and rapid analysis time. Indeed, these advantages have led to significant interest in EMMA methodology; however, most of the interest has been focused toward the development of enzymatic analyses (*23*−*25*) where enzyme turnover can compensate for the low concentration sensitivity that is inherent to CE systems with UV absorbance detection. Still, an increasing number of reports of EMMA with small molecule reactions have recently appeared (*26*−*28*), and these have been primarily in areas in which high micromolar to millimolar concentrations of analytes are possible. Because TAC values are often in the millimolar range, CE analyses with inline UV detection should have more than adequate sensitivity. Consequently, the determination of TAC with EMMA becomes an intriguing possibility.

In this work, we investigate the utility of EMMA to determine TAC values of aqueous samples containing antioxidants and demonstrate proof-of-concept for this approach using a limited set of commercial beverages. We use 2,6-dichlorophenolindophenol (DCIP), a common redox indicator dye molecule, as the oxidizing agent in a rapid inline electron transfer reaction with antioxidants. DCIP is an attractive choice of oxidizing agent as it (i) is easily detected by UV−vis absorbance in either redox form, (ii) has redox state sensitive spectral properties, (iii) exhibits fast electron transfer kinetics, and (iv) has reasonable mobility so that the individual redox forms of DCIP can be easily separated with CE distances and voltages without significant electrodispersion. The reported inline method is applied to assess the TAC of a variety of commercial juice/beverage samples, and the results are compared to those obtained with the accepted FRAP method. The effect of sample conductivity on the EMMA results is also investigated.

MATERIALS AND METHODS

Reagents. Sodium phosphate monobasic, L-ascorbic acid, and 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) were purchased from Sigma-Aldrich Co. (St. Louis, MO). 2,6-Dichlorophenolindophenol sodium salt (DCIP) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Ferric chloride was purchased from J. T. Baker Chemical Co. (Philipsburg, NJ), glacial acetic acid was from EM Science (Gibbstown, NJ), and sodium hydroxide and hydrochloric acid were from Fisher Scientific (Fair Lawn, NJ).

Buffer Solutions. Phosphate buffer (50.0 mM) was prepared by dissolving monobasic sodium phosphate in 18 MΩ·cm water. The pH was adjusted to 7.20 using 1 M NaOH and an Accument pH-meter (Fisher Scientific). The acetate buffer used in the FRAP experiments was prepared by diluting glacial acetic acid with 18 MΩ·cm water and adjusting the pH to 3.60 with aqueous sodium hydroxide. All buffer solutions were filtered through 0.45 μm PTFE syringe filters into 1 mL polypropylene vials (Agilent Technologies, Santa Clara, CA) before use. Buffer solutions were prepared weekly and stored at 4 °C when not in use.

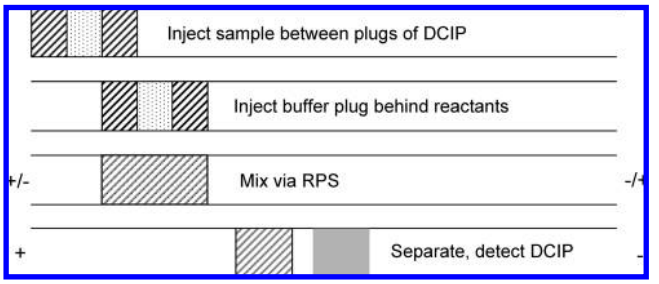

Figure 1. Capillary diagram outlining the process for determining the total antioxidant capacity of a beverage sample by electrophoretically mediated microanalysis (EMMA) with 2,6-dichlorophenolindophenol (DCIP). Beverage sample plug is dotted, DCIP reagent plug is upward diagonal, and reduced DCIP product plug is solid gray.

Calibration Standards. Standard and analysis solutions of the antioxidant ascorbic acid were prepared daily in degassed 18 MΩ·cm water with, in some cases, 0−100 mM NaCl added to adjust the sample conductivity. All solutions were filtered through a 0.45 μm PTFE syringe filter (JSI Scientific, Freehold, NJ) prior to use, and stored at 4 °C when not in use.

Commercial Beverage Samples. Seven commercial beverage samples were obtained at a local retail outlet. Samples were filtered through a 0.45 μm PTFE syringe filter (JSI Scientific) and diluted with either 18 MΩ·cm water or an aqueous NaCl solution prior to use. The beverage samples included Minute Maid apple juice (FEB0909 AM65 2126 CT809), Ocean Spray 100% cranberry juice (CT981 0507 PCJ), Welch's 100% grape juice (NE08104 20:33 P), and four different Glaceau Vitamin Water products: XXX acai−blueberry−pomegranate (B8206 14:02 A CT795), Formula 50 grape (B8164 10:10 B CT735), Rescue green tea (B8148 19:39 B CT735), and multi-v lemonade (B8140 22:37 C CT735). All analyses of beverage samples were carried out on the same day that the samples were first opened as many samples suffered from significant air oxidation upon overnight storage after being opened.

Equipment. An Agilent 3D capillary electrophoresis system (Agilent Technologies, Santa Clara, CA) with photodiode array UV−visible detection and Agilent Chemstation software was used for all separations. An HP 8452A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA) controlled by HP 89530A UV−vis operating software on a PC were used for FRAP analysis.

EMMA Procedure with DCIP. A 50.0 μm i.d. unmodified fused silica capillary (Polymicro Technologies, Phoenix, AZ) 33.0 cm in length (24.5 cm effective) was used for all EMMA analyses. The running buffer was 50.0 mM phosphate at pH 7.20, and the capillary was maintained at a constant temperature of 25.0 °C. Absorbance detection was performed at 590, 265, and 210 nm, each with an 8 nm bandwidth. A 2.00 min flush with run buffer was performed between runs. Each day, capillaries were reconditioned with 2.00 min flushes of 1.0 M NaOH, 18 MΩ·cm water, and the running buffer, sequentially.

The EMMA procedure involved the use of the oxidizing agent DCIP as outlined in **Figure** 1. A 150 mbar·s injection of sample was sandwiched between two 150 mbar·s injections of 0.60 mM DCIP. A 300 mbar·s plug of buffer was injected behind the analytes to guard against loss of reagent to the inlet vial. Following the injection sequence, an alternating potential of ±15.0 kV (2.7 s at each polarity, 10 iterations), referred to as rapid polarity switching (RPS) (*29*), was applied to mix the antioxidants with DCIP. After the RPS step, a 10.0 kV separation potential was applied, to separate the remaining DCIP and the reduced DCIP product via capillary zone electrophoresis (CZE). L-Ascorbic acid was chosen as the calibration standard. For the analysis of commercial beverages, 400 μL of 5-fold diluted beverage was mixed with x μL of 1.00 mM ascorbic acid standard and $(400 - x)$ μL of 18 MΩ·cm water. All samples were vortex mixed in polypropylene samples vials (Agilent Technologies, Palo Alto, CA) prior to being placed in the sample tray.

FRAP Procedure. The FRAP assay was performed using a modified version of the procedure detailed by Benzie and Strain (*8*). The FRAP reagent consisted of a 10:1:1 ratio of solutions of acetate buffer, ferric chloride, and TPTZ. The acetate buffer system was 100 mM at pH 3.6. The ferric chloride solution was 20.0 mM, prepared in 18 MΩ·cm water. The TPTZ solution was 10.0 mM, prepared in 40.0 mM HCl. FRAP calibration was accomplished via analysis of solutions consisting of 900 μL of FRAP reagent and a varied amount (x μL) of 0.500 mM ascorbic acid, with an appropriate volume (100 − x μL) of 18 MΩ·cm water. For the analysis of commercial beverages, 100 μL of 10-fold diluted beverage was added to 900 μL of the FRAP reagent solution. The FRAP procedure was carried out at room temperature in a 0.50 cm path length disposable cuvette, and the absorbance of the product at 594 nm was recorded every 10 s for a period of 10 min. The final absorbance reading was used for quantification.

RESULTS AND DISCUSSION

A schematic representation of the use of EMMA for the determination of TAC is shown in **Figure** 1. The EMMA method employed here involves a sandwich assay in which a plug of approximately 9 nL of the antioxidant-containing sample is injected between plugs of a reagent solution containing an excess of DCIP. When mixed, an ET reaction between DCIP and the antioxidant(s) in the sample causes the formation of dichlorodihydroxydiphenylamine, the reduced form of DCIP. The system can be calibrated using ascorbic acid, a commonly used antioxidant standard for aqueous systems that is known to react rapidly with DCIP with 1:1 stoichiometry (*30*, *31*). At neutral pH, DCIP carries a negative charge (*32*), allowing its two redox forms to be easily separated from one another and from other anions in a sample via electrophoresis. DCIP also has a strong chromophore that is conveniently sensitive to oxidation state, making it an attractive choice for wavelength-selective detection of the individual redox forms. Sandwiching the sample between plugs of DCIP establishes conditions under which sample antioxidants, regardless of charge, will mix with the DCIP reagent when the RPS mixing potential is initiated. The dual plug arrangement also allows for bidirectional diffusional mixing of any neutral antioxidants that may be present in the sample.

Typical electropherograms obtained via the in-capillary TAC analysis of an aqueous standard of L-ascorbic acid are given in **Figure** 2. A broad neutral peak marking the flow of the sample matrix past the detector is observed at about 2.3 min, whereas the analytically useful DCIP peaks are observed at later times. The anionic nature of DCIP allows both the original reagent and its reduced product to be electrophoretically separated from other sample components. The excess DCIP reactant, which under the conditions employed migrates upstream with an electrophoretic mobility of ca. -2.1×10^{-4} cm^2 V^{-1} s^{-1}, absorbs strongly at 590 nm (peak 3 in **Figure** 2**A**), where many potential interfering ions will not absorb. The reduced DCIP product (peak 2, $\mu_{ep} = -1.5 \times 10^{-4}$ cm^2 V^{-1} s^{-1}) has a local absorbance maximum at 265 nm and can be detected with greater sensitivity at this wavelength (**Figure** 2**B**). Both forms can be sensitively detected at very low wavelength (210 nm, **Figure** 2**C**), but at this wavelength there is a greater risk of comigrating sample ions interfering with quantification (see **Figure** 6).

To explore the linearity of response, standard aqueous solutions of L-ascorbic acid were analyzed with the EMMA methodology. As seen in **Figure** 3, increasing the concentration of ascorbic acid (bottom to top) in the sample plug leads to decreasing peak areas for DCIP and correspondingly increasing peak areas for the reduced product. The calibration data for the decreasing DCIP peak area at 590 nm, its λ_{max}, were described by $y = (-370 \pm 10)x + (642 \pm 6)$ with $r^2 = 0.997$. Calibration data using the appearance of the reduced DCIP product peak areas at 265 nm were $y = (132 \pm 2)x - (1.3 \pm 1.2)$ with $r^2 = 0.9991$. Thus, the inline reaction scheme can be calibrated via the disappearance of the DCIP, as well as by the appearance of the reduced DCIP product; however, the RSDs at 590 (≤2.5%) were uniformly superior to those at 265, which were as high as 10% with the lowest concentration samples. Still, both 590 and 265 nm data were monitored with real samples (i) to ensure that DCIP was not completely depleted and (ii) as a control for the possibility of bias from an anion from the sample comigrating with DCIP. We did not encounter the latter case with any of the samples analyzed.

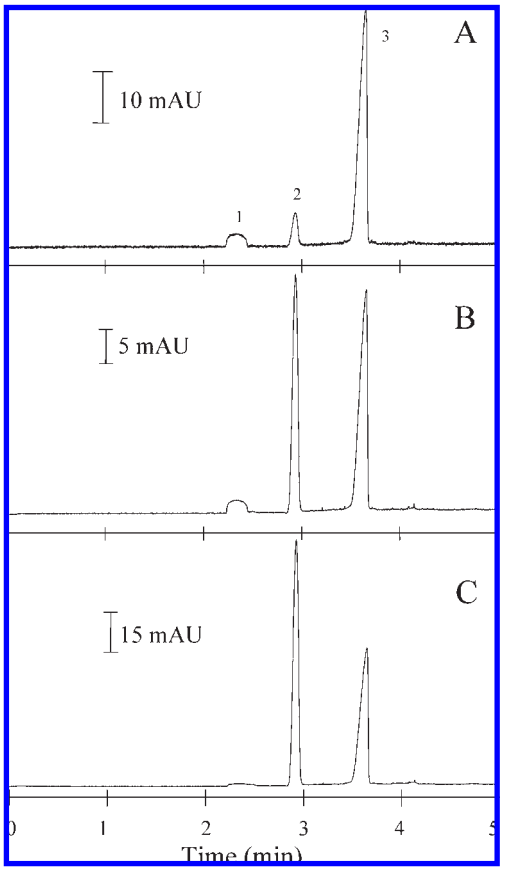

Figure 2. Typical raw data for inline TAC analysis of a 0.60 mM aqueous standard of ascorbic acid as observed at 590 nm (**A**), 265 nm (**B**), and 210 nm (**C**). Peaks: 1, neutral marker; 2, reduced DCIP product; 3, excess DCIP reagent. Conditions were as given under Materials and Methods.

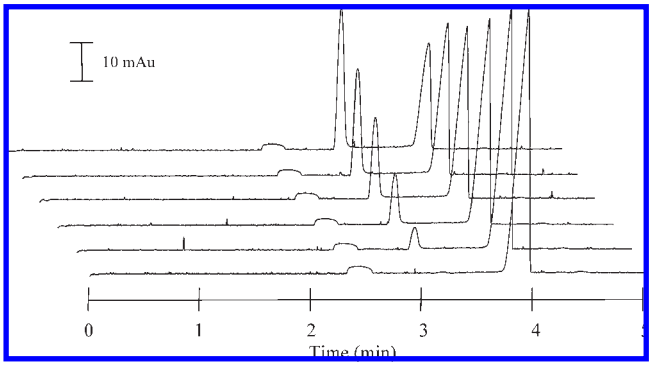

Figure 3. Raw data for inline TAC analyses of varied ascorbic acid standards prepared in 18 MΩ·cm water and ranging in concentration from 0 mM (bottom trace) to 1.00 mM (top trace) in 0.20 mM increments. Time axes are offset for clarity; each separation is 5.00 min in duration. Detection was at 265 nm. Other conditions were as stated under Materials and Methods.

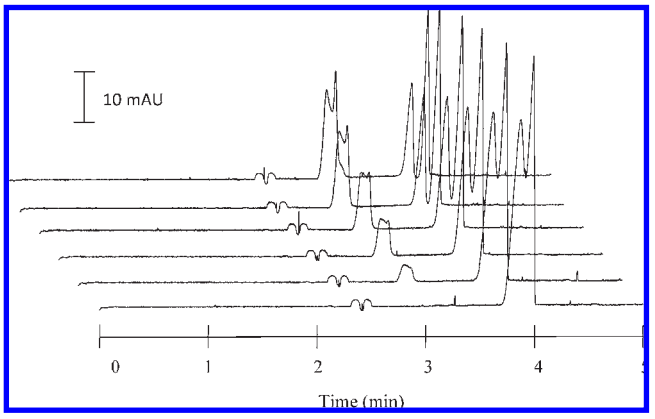

Figure 4. Inline TAC analyses of varied ascorbic acid standards prepared in 100 mM NaCl and ranging in concentration from 0 mM (bottom trace) to 1.00 mM (top trace) in 0.20 mM increments. Time axes are offset for clarity; each separation is 5.00 min in duration. Detection was at 265 nm. Other conditions were as stated under Materials and Methods.

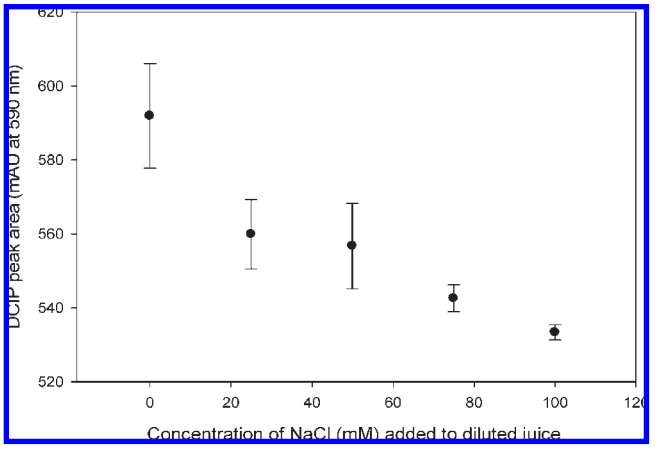

Figure 5. Plot of DCIP reagent peak areas at 590 nm obtained with inline TAC analysis of 10% apple juice with various amounts of NaCl added as a matrix modifier. A signal change of about 10% is apparent over this range. Error bars represent standard deviation (n = 3).

The analytical response of the reported method to the polyphenolic antioxidant catechin was also examined. The method gave linear and reproducible results for this large antioxidant molecule, but the calibration slope with catechin was considerably lower than with ascorbic acid, indicating differing response factors for these two antioxidants. With the EMMA method presented, the calibration data for catechin, using the signal for DCIP at 590 nm, is described by $y = (-96 \pm 3)x + (650 \pm 2)$ ($r^2 = 0.996$). That is, the sensitivity of this method for catechin was only about 26% of that seen with ascorbic acid. The lower response factor for catechin is most likely due to a combination of less favorable thermodynamic factors (lower $E°$ value for the redox half reaction) and slower electron transfer kinetics. Differing response factors is one of the necessary compromises one makes when measuring TAC, regardless of the methodology employed, and the reported EMMA methodology presented here is also subject to this limitation. Indeed, significant and sometimes drastically different values for TAC have been reported for analyses of the same samples with published methods for TAC determination (*11*−*14*). Nonetheless, TAC remains a useful measure of the aggregate ability of a given sample to adjust to oxidative assault, and the method reported here is shown to respond linearly, albeit with different slope, to two very different antioxidants.

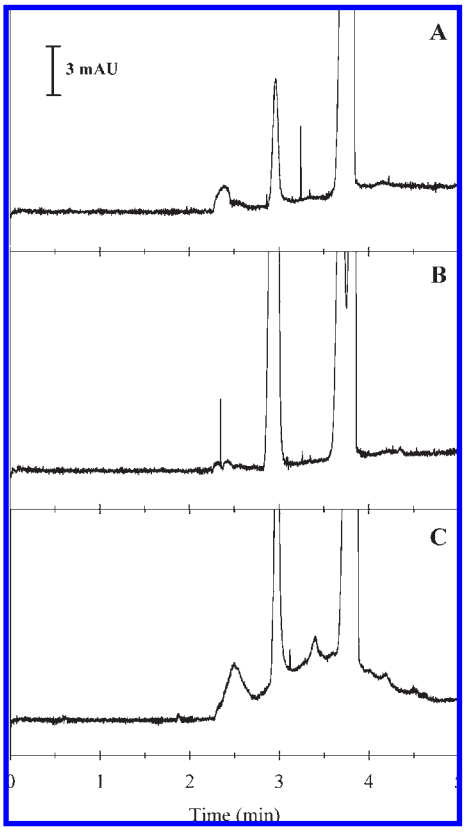

Figure 6. Expanded view of baseline (at 210 nm) for the inline TAC analysis of 10-fold diluted cranberry juice (**A**), a 0.60 mM ascorbic acid standard in 100 mM NaCl (**B**), and 10-fold diluted grape juice (**C**). The minor interference observed at 210 nm is not seen at 590 nm.

One of the important aspects to consider with any electromigration method is the salt content of the sample. Because the conductivity of the sample can affect the local voltage field within the sample plug, and therefore the initial migration velocity of ions in that environment, varied ionic content between standard and sample matrices can be problematic. With conventional CE separations, samples are often prepared in low ionic strength solutions so ionic samples will concentrate or "stack" at the sharp boundary in the field strength that develops where sample matrix and the background buffer meet. Conversely, a deconcentrating effect can occur when very conductive samples are injected. The effect of sample salt on performance with the inline EMMA system does not follow this trend. The reaction between DCIP and ascorbate was investigated by analyzing a series of ascorbic acid standards that also contained 100 mM NaCl (**Figure** 4). The calibration data remained linear ($y = (137 \pm 2)x - (6.0 \pm 0.9)$, $r^2 = 0.9997$), and reproducibility was not compromised. However, the DCIP response is no longer detected as a single peak, but rather as two overlapping peaks corresponding to the discrete plugs of this reagent that were injected but did not merge into a single zone by the time they reached the detection point. That is, the field within the sample was too low to allow the DCIP to fully traverse the sample and merge with the second plug of DCIP prior to detection. In addition, the reduced DCIP peak widened and also became somewhat bimodal. The summed peak areas from the two peaks (or single bimodal peak) still indicate good linearity, and the absence of an ascorbic acid peak indicates that the reaction between DCIP and ascorbic acid appears to go to completion with or without salt in the sample. However, the initial migration dynamics of DCIP are clearly altered. Without salt in the sample, the applied separation potential creates a

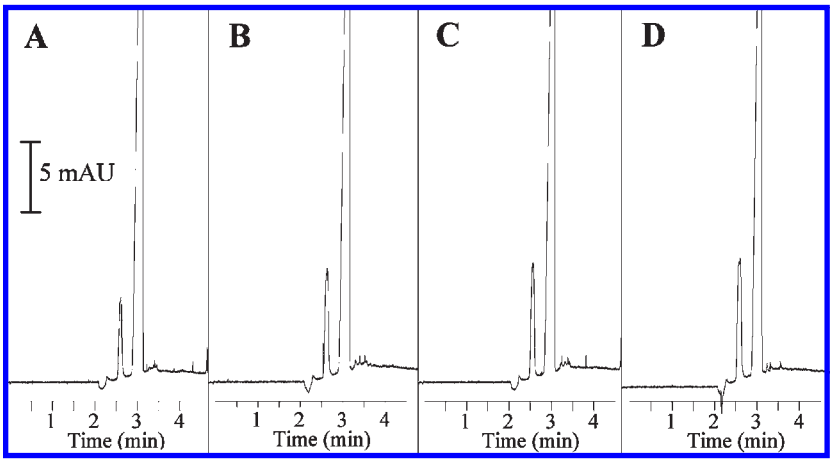

Figure 7. Raw data at 265 nm for the inline TAC analysis of 10-fold diluted cranberry juice (**A**) and the same juice spiked with 0.10 mM (**B**), 0.20 mM (**C**), and 0.30 mM (**D**) ascorbic acid. The smaller peak at ca. 2.5 min in each frame is the reduced DCIP product, whereas the larger peak at ca. 3 min is the unreacted DCIP reagent.

strong voltage field within the resistive sample plug and the anionic DCIP migrates rapidly through the resistive sample matrix and merges with the DCIP anions on the other side of the sample plug. When NaCl is added to the standards, the samples have higher conductivity, which slows the migration velocity of DCIP within the sample plug, and two zones of DCIP are evident at the detection point.

One must be careful to address the potentially troublesome effect of varied sample ionic strength on quantifications of TAC. One should be cautioned against external calibration with aqueous (no salt) ascorbic acid standards; this approach gave rise to apparent percent recoveries of ascorbic acid that varied from 65 to 110%. To probe the range and magnitude of the effect of sample conductivity, varied amounts of salt were added to an apple juice sample, and gradually lower DCIP peak areas were obtained with increased salt content. The decrease in area amounted to only about a 10% difference between no salt and 100 mM salt added to the sample (**Figure** 5). Higher amounts of salt had little additional effect, presumably because the conductivity of the sample had reached that of the background buffer (at 100 mM NaCl). However, a modest increase in reproducibility was achieved by adding salt to the samples.

While the problems associated with varied sample conductivity could be minimized by adding a large amount of salt to *all* samples and standards, the method of standard additions is perhaps the best approach to quantification. Several commercial juice samples were analyzed with the EMMA approach using the method of multiple standard additions to obtain quantitative data. Some minor baseline disruptions were observed in the region of interest with some samples at 210 nm (**Figure** 6), and these were avoided by performing detection at 265 nm. Typical raw data obtained at 265 nm for a juice sample spiked with various amounts of ascorbic acid is given in **Figure** 7. For all samples analyzed, the product peak area at 265 nm exhibited linearity with the volume (0, 80, 160, and 240 μL) of 1.00 mM ascorbic acid addition to the 800 μL total sample volume, but the slope of resultant data varied from 0.070 to 0.206, depending on the sample. The resulting quantitative determinations of TAC, expressed as millimolar equivalents of ascorbic acid, are presented in **Table** 1. The same beverage samples were also analyzed with the commonly used FRAP assay, and despite the differing redox chemistry and solution pH between the two methods, the TAC values are surprisingly comparable. It is perhaps not too surprising that the samples most likely to have polyphenolic compounds (grape juice and green tea) show the largest deviations between the two techniques. It appears from this limited, but diverse, set of samples that the rapid and easily automatable EMMA methodology, used in conjunction with the method of standard additions, may be a viable alternative to more cumbersome methods to determine TAC.

Table 1. Comparison of Measured Values for Total Antioxidant Capacity (Expressed in Milliequivalents of Ascorbic Acid per Liter, mequiv of AA/L) of Commercial Beverage Samples Using the Reported EMMA DCIP Method and the Widely Used FRAP Assay[a]

	total antioxidant capacity (mequiv of AA/L)	
sample	EMMA[b]	FRAP[c]
Formula 50	1.4 ± 0.5	1.8 ± 0.2
Multi-V	2.3 ± 0.4	2.03 ± 0.13
XXX	2.83 ± 0.16	3.9 ± 0.2
green tea	3.12 ± 0.14	5.3 ± 0.4
apple juice	4.4 ± 1.1	4.4 ± 0.2
cranberry juice	3.2 ± 0.6	3.9 ± 0.3
grape juice	3.3 ± 0.3	nd[d]

[a] Juice samples were diluted 10-fold prior to analysis. Errors represent standard deviation (n = 3). [b] EMMA analysis with DCIP was carried out via multiple standard additions of ascorbic acid; uncertainties represent propagated uncertainty from best fits of triplicate analysis. [c] FRAP analysis was carried out by kinetic method based on ref *8* with external calibration with ascorbic acid standards; uncertainties represent standard deviation of triplicate analyses of each individual sample. [d] The grape juice sample had appreciable absorbance at 594 nm, masking determination of TAC by the FRAP method.

In conclusion, the use of the oxidant DCIP as an in-capillary reactant appears to be a reasonable alternative to published methodologies for assessing the TAC. The EMMA methodology requires minimal amounts of sample and generates negligible waste while providing quantitative data that are well within the range of variance typically seen when TAC methods are compared. Owing to differences in ionic strength in commercial samples, a spiking approach to quantification appears to be advisible. Overall, this new inline methodology shows reasonable promise as a rapid and low-volume alternative to the current TAC methodology.

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