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Separation of open-cage fullerenes using nonaqueous capillary electrophoresis☆

Hsiu-Li Su, Wan-Chun Kao, Cheng-yu Lee, Shih-Ching Chuang, You-Zung Hsieh*

Department of Applied Chemistry, National Chiao Tung University, 1001 Ta Hsueh Road, Hsinchu 300, Taiwan

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ABSTRACT

In this study, nonaqueous capillary electrophoresis (NACE) was used to separate three open-cage fullerenes. Trifluoroacetic acid (TFA) was used as the nonaqueous background electrolyte to change the analytes' mobilities. The selectivity and separation efficiency were critically affected by the nature of the buffer system, the choice of organic solvent, and the concentrations of TFA and sodium acetate (NaOAc) in the background electrolyte. The optimized separation occurred using 200 mM TFA/20 mM NaOAc in MeOH/acetonitrile (10:90, v/v), providing highly efficient baseline separation of the open-cage fullerenes within 5 min. The migration time repeatability for the three analytes was less than 1% (relative standard deviation). Thus, NACE is a rapid, useful alternative to high-performance liquid chromatography for the separation of open-cage fullerenes.

1. Introduction

Open-cage fullerenes are fascinating studies because of their unusual structures and properties. For example, because they can trap and release guest species reversibly, they can be used for molecular storage [1]. Since Wudl [2] synthesized the first open-cage fullerene, *N*-MEM-ketolactam, several papers have described various other types of fullerene derivatives [3–6]. The presence of a surface orifice in an open-cage fullerene allows the incorporation of an atom, a small molecule, or an ion to form an endohedral fullerene complex. The orifice size is an important factor affecting the size of the guest species; the encapsulation of a molecule requires a sufficiently large orifice [4]. The orifice size can be changed by varying the number of atoms in the rim (e.g., 16- or 20-membered-ring orifices) [5] or by inserting a larger element into the rim (e.g., such as replacing a sulfur atom with a selenium atom) [6]. Because of the structural complexity of the various open-cage fullerene derivatives, it is necessary to develop appropriate techniques for their separation and identification.

The analysis of open-cage fullerenes using chromatographic methods is a challenging task because of their low abundances, poor solubilities in water, and similar structures [7]. HPLC has been used widely for the separation of these compounds, with many stationary phases – including phenothiazinyl silica (Buckyprep-M), pyrenylpropyl silica (Buckyprep), and pentabromobenzyl silica (PBB) – specially designed for the separation of metallofullerenes, fullerenes, and fullerene derivatives [8]. Although HPLC methods can allow the separation of open-cage fullerenes, the consumption of the organic solvent used as mobile phase is large. In contrast, methods based on capillary electrophoresis (CE) have the advantages of rapid analysis times, high separation efficiencies, low solvent consumptions, and low sample volumes, making it suitable for the development of new tools for the separation of open-cage fullerenes.

The choice of the separation medium for CE is critical; in most cases, aqueous media provide sufficient separation efficiencies, except when the analytes (e.g., open-cage fullerenes) are insoluble in water, but soluble in organic solvents. Nonaqueous capillary electrophoresis (NACE) is the name given to the technique when the separation medium comprises totally (or predominantly) an organic solvent [9–12]. In addition to its major advantage (the ability to analyze poorly water-soluble compounds), NACE also exhibits several unusual and interesting features [9,10]. First, the wide-ranging physical and chemical properties of organic solvents allow fine-tuning of a number of operating parameters to improve selectivity [11]. Second, nonaqueous background electrolytes usually produce lower electric currents than do aqueous electrolytes; this phenomenon decreases the Joule heat problem, allowing higher voltages to be applied, thereby resulting in rapid analyses with high peak efficiencies [12]. Third, NACE is more suited than aqueous CE for application to hyphenated systems with mass spectrometric detection, thereby extending its applicability. Finally, NACE can be used to separate nanomaterials [13] and charged and uncharged compounds [14–17]: neutral analytes can be separated after deprotonation, protonation, or heteroconjugation [10].

Aqueous CE is the most commonly used method for the electrophoretic separation of fullerenes [18,19]. To overcome solubility problems, the fullerenes are typically dissolved in aqueous media

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* Corresponding author. Tel.: +886 3 5731785; fax: +886 3 5723764.
E-mail address: yzhsieh@mail.nctu.edu.tw (Y.-Z. Hsieh).

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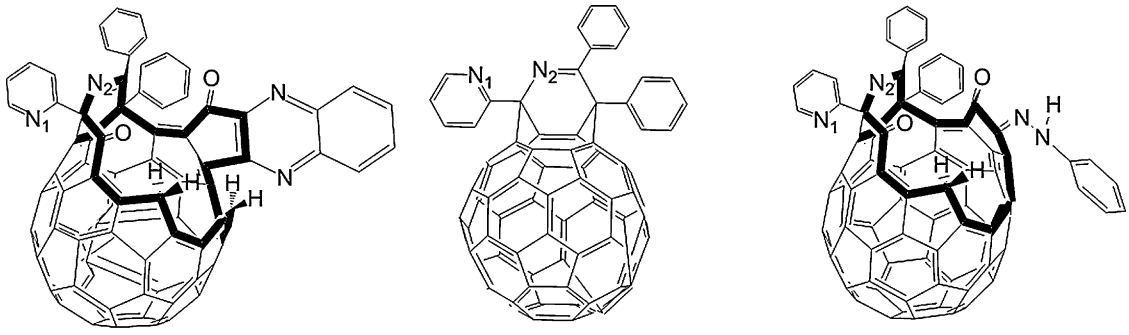

1 2 3

Fig. 1. Molecular structures of the three open-cage fullerenes.

through derivation [20] or complexation with γ-cyclodextrin [21], lipids [22], or surfactants [23], thereby appending an additional time-consuming sample preparation step to the analysis procedure. Although fullerenes are more soluble in organic solvents, to the best of our knowledge NACE has been reported only once for fullerene separation, when Wan et al. separated C_{60}, C_{70}, and C_{84} using a NACE method depending on the solvophobic interactions between tetraalkylammonium ions and the analytes [7]. Thus, we were motivated to develop a new NACE method for the separation of three open-cage fullerenes.

Fig. 1 displays the structures of the selected open-cage fullerenes. Compound **1**, which bears a 20-membered-ring orifice, can trap water molecules. Compound **2** features an 8-membered-ring orifice. Compound **3**, which contains a 16-membered-ring orifice, can trap H_2 molecules. Each of these compounds features several basic nitrogen atoms; therefore, protonation would increase the analytes' charge and change their migrating behavior. In this study, we investigated the influences of the composition of the organic solvent, the nature of the acid and its concentration, and the concentration of the background electrolyte. We then optimized the separation conditions to obtain the best resolution for the qualitative and quantitative analyses of open-cage fullerenes.

2. Experimental

2.1. Chemicals and reagents

All reagents and chemicals were of analytical grade. Formic acid was purchased from Acros Organics (Geel, Belgium). Acetic acid (AcOH) and acetonitrile (ACN) were obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA). Trifluoroacetic acid (TFA) was obtained from Alfa Aesar (Ward Hill, MA, USA). Toluene was purchased from Osaka Organic Chemical Industry (Osaka, Japan). Sodium hydroxide (NaOH) was purchased from Fluka (Buchs, Switzerland). Sodium formate, sodium acetate (NaOAc), and hydrochloric acid (HCl) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol (MeOH) was purchased from Echo Chemical (Miaoli, Taiwan). Water was purified through a Millipore Milli-Q water system (Milford, MA, USA). The syntheses of the fullerene derivatives **1**–**3** have been described previously [4]; they were characterized using nuclear magnetic resonance (NMR), infrared (IR), and ultraviolet (UV) spectroscopies and mass spectrometry (MS).

2.2. Apparatus

A Beckman P/ACE MDQ CE system (Fullerton, CA, USA) was used to effect the separations. A diode-array detector was employed for detection. Separations were performed in a 50-cm (effective length: 40 cm) × 50-μm I.D. fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA), which was assembled in cartridge format. A personal computer using 32 Karat software controlled the P/ACE instrument and allowed data analysis. Samples were pressure-injected at 2.07 kPa for 3 s. The detection wavelength was set at 200 nm. The separation proceeded with a positive applied potential (25 kV). Prior to use, the separation capillary was preconditioned sequentially with MeOH (10 min), 1 M HCl (10 min), deionized water (2 min), 1 M NaOH (10 min), and then deionized water again (2 min). Between runs, the capillary was flushed sequentially with 1 M NaOH (10 min), water (5 min), and the background electrode (BGE) solutions (2 min).

Stock standard solutions (500 μg/mL for compound **1** and 400 μg/mL for compounds **2** and **3**) were prepared in toluene and refrigerated at 4 °C. Prior to analysis, each stock solution was diluted to 20 μg/mL using a BGE to form the working solution. The BGEs were prepared in 100–250 mM TFA, 10–30 mM NaOAc, and MeOH/ACN (from 10:90 to 50:50, v/v). Calibration curves were obtained after preparing standard solutions of the three open-cage fullerenes individually at 2.5, 7.5, 15, 25, and 35 μg/mL. Experiments were performed five times at each concentration.

3. Results and discussion

3.1. Selection of nonaqueous background electrolyte solution

The most effective method for changing the selectivity of the separation of these open-cage fullerenes is to affect their ionization. Because compounds **1**–**3**, all feature basic nitrogen atoms, protonation would impart them with positive charge and, thereby, change their migrating behavior. In a preliminary experiment, we examined the ability of three nonaqueous BGEs – formic acid/sodium formate, AcOH/NaOAc, and TFA/NaOAc – to modify the degree of analyte protonation. Fig. 2 reveals that the three compounds appeared in front of the electroosmotic flow (EOF) in the 200 mM TFA/20 mM NaOAc BGE, but migrated with the EOF in the 200 mM formic acid/20 mM sodium formate BGE and in the 200 mM AcOH/20 mM NaOAc BGE. These results indicate that the nitrogen atoms of the analytes were protonated by TFA, but not by the other two acids; note that the pK_a of TFA (0.3) is lower than those of formic acid (3.7) and AcOH (4.8). In NACE, the nature of the organic solvent influences the pK_a of both the electrolytes and analytes. In general, the values of pK_a of acidic compounds (HA) are dramatically higher in organic solvents (e.g., the pK_a of AcOH increases from 4.8 in H_2O, to 9.7 in MeOH, and further to 22.3 in ACN), whereas the effects on basic compounds (HB^+) [11] are relatively minor. Therefore, these acids possessed elevated values of pK_a in the organic solvents, but the values of pK_a of the analytes were relatively unchanged. Our results revealed that the BGE had an effect on the degree of proto-

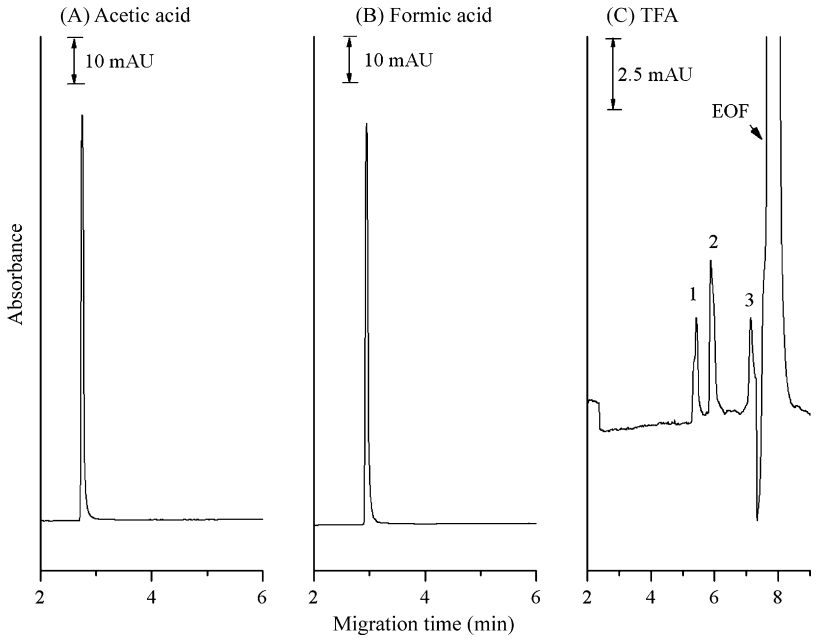

Fig. 2. Electropherograms of the three analytes in BGEs of MeOH/ACN (25:75, v/v) containing (A) 200 mM AcOH/20 mM NaOAc, (B) 200 mM formic acid/20 mM sodium formate, and (C) 200 mM TFA/20 mM NaOAc. Sample injection at 2.07 kPa for 3 s; sample concentration, 20 μg/mL. Peak identification: (1) compound **1**; (2) compound **2**; (3) compound **3**.

nation of the analytes. The values of pK_a of formic acid and AcOH differ by almost one order of magnitude in water, but they exhibited similar effects on the analytes in MeOH/ACN (25:75, v/v). Nevertheless, the analytes could be separated in the BGE containing TFA, due to their different degrees of protonation.

Fig. 2(C) indicates that compounds **1**–**3** appeared before the EOF emerged and migrated sequentially in the 200 mM TFA/20 mM NaOAc BGE. This result suggests that each analyte bore a positive charge and that compound **1** had the largest effective mobility, which is proportional to its charge (q) and solvation size (r). Although it is difficult to determine the exact values of pK_a of the analytes, their chemical structures provide us with some clues. The extent of protonation of the nitrogen atoms N_1 and N_2 in the three compounds should be similar. There are two other nitrogen atoms that could be protonated in compound **1**; one is a pyridine nitrogen atom and the other is part of the imine functionality. Although compound **1** has a larger size than compounds **2** and **3**, it also possesses greater effective mobility. Compound **2** has only two nitrogen atoms that could be protonated, i.e., two fewer than compound **1**. Although compound **3** also has four nitrogen atoms in its structure, two of them are on the hydrazone unit, which would be difficult to protonate. Hence, we might expect compound **3** to be protonated to a similar extent as compound **2**, but its larger size would decrease its relative mobility. Therefore, the order of migration of compounds **1**–**3** is consistent with their ratios of charge/solvation size.

3.2. Effect of MeOH/ACN ratio on separation

We studied the effect of the content of MeOH (10–50%, v/v) in the MeOH/ACN mixtures on the separation of the open-cage fullerenes (while keeping the electrolyte concentration constant). Fig. 3 reveals that the migration times of analytes increased upon increasing the MeOH content, primarily because of the decreased EOF mobility (see the inset of Fig. 3). The EOF mobility in NACE is directly proportional to the ratio between the solvent's dielectric constant and viscosity (ε/η) and is a function of the zeta potential of the capillary surface [18]. MeOH/ACN mixtures are used widely in NACE to impart various physical properties to the nonaqueous electrolyte solution, including changing the EOF. When Vaher and Koel studied the relationship between the EOF mobility and the relative contents of a binary solvent system (MeOH/ACN), they found that the decrease in EOF mobility was not linear with respect to the percentage of MeOH [24]. Similarly, in our experiment, increasing the MeOH percentage resulted in enhanced separation of the three analytes, but it broadened the peaks and increased the analysis times. These phenomena are probably due to the decreased EOF mobility. Nevertheless, the addition of 10% MeOH shortened the analysis time while maintaining full resolution for all compounds. Thus, taking into account the analysis time and resolution, a MeOH content of 10% was used as an optimized condition for our subsequent experiments.

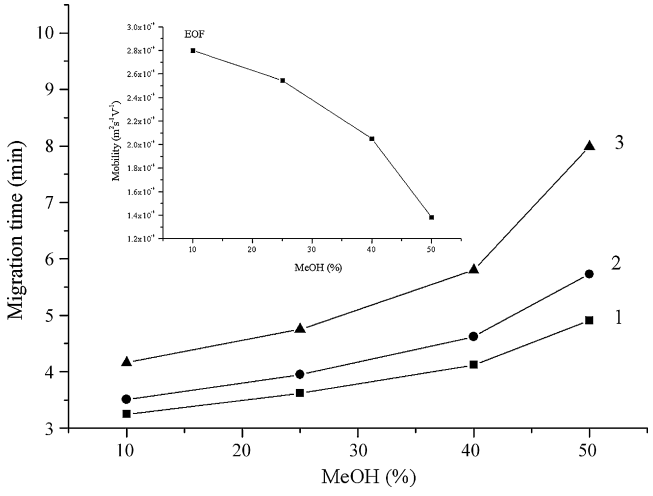

Fig. 3. Plots of the migration times of the three analytes with respect to the MeOH content in BGEs comprising 200 mM TFA/20 mM NaOAc in MeOH/ACN mixtures of various proportions (inset: EOF mobility plotted with respect to the MeOH content). Other conditions were the same as those used to obtain Fig. 2.

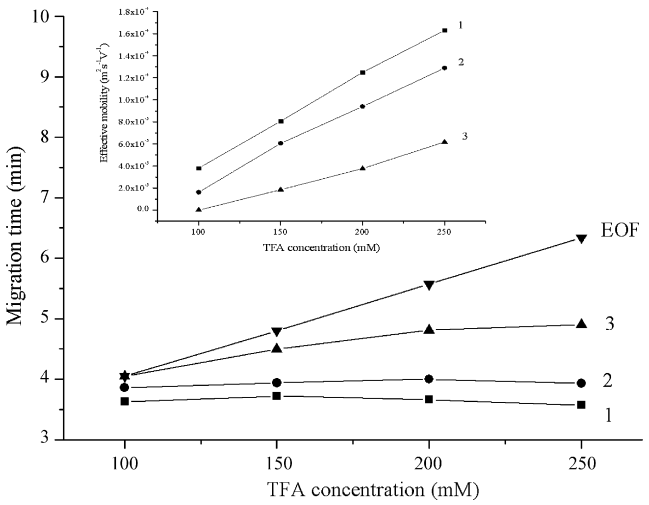

Fig. 4. Plots of the migration times of the EOF and the three analytes with respect to the TFA concentration in a BGE of 20 mM NaOAc in MeOH/ACN (10:90, v/v). Inset: Effective mobilities of the three analytes plotted with respect to the TFA concentration. Other conditions were the same as those used to obtain Fig. 2.

3.3. Effect of TFA concentration on separation

Fig. 4 displays the effect of the TFA concentration in the BGE on the separation of the open-cage fullerenes. TFA was added within the range from 100 to 250 mM to the optimal MeOH/ACN mixture (10:90, v/v). The inset of Fig. 4 reveals that the effective mobilities of all of the compounds increased upon increasing the TFA concentration, presumably because enhanced degrees of protonation increased the analytes' mobilities. Nevertheless, the mobility of the EOF decreased upon increasing the TFA concentration. As a result, the increasing effective mobilities of the analytes and the decreasing EOF mobility resulted in similar migration times for analytes **1** and **2** when the TFA content was increased from 100 to 250 mM. For subsequent experiments, we selected a TFA concentration of 200 mM because it provided good peak shapes, an acceptable analysis time, and adequate resolution for all of the compounds.

3.4. Effect of NaOAc concentration on separation

Fig. 5 displays the effect of the NaOAc concentration on the separation of the three analytes at constant values of the TFA concentration and the organic solvent contents. When the NaOAc concentration increased, the migration times of all of the compounds increased. At 10 mM NaOAc, the short analysis time led to poor separation of compounds **1** and **2**, suggesting possible errors in quantification. At 30 mM NaOAc, the increased analysis time resulted in low resolution of compound **3** and the EOF marker. Taking into account the optimized resolution for all compounds and the good peak shapes obtained when 20 mM NaOAc was present in the BGE, we employed this concentration for our subsequent quantitative analyses.

3.5. Effect of applied voltage, capillary temperature, and sample injection time on separation

The effect of applied voltage on the separation was investigated at applied voltages of 20, 25, and 30 kV. When the applied voltage increased, the migration times of the compounds decreased and the generated electric current increased (from 20 to 30 μA). Increasing the analytes' electrophoretic mobilities led, however, to poor separation of compounds **1** and **2** at 30 kV. Conversely, the total analysis time was the longest at 20 kV. Considering these results, an applied voltage of 25 kV (with the generated electric current of 25 μA) was selected for subsequent separations.

The effects of the capillary temperature on the separation efficiency, electric current, and migration time were studied at 20, 25, and 30 °C. When increasing the temperature, the migration times of the analytes decreased upon slightly increasing the electric current; in addition, varying the capillary temperature had minor effects on the resolution of the analyte signals. At an elevated capillary temperature of 30 °C, however, evaporation of the solvent became a significant concern, resulting in poor repeatability. Thus, taking into account the analysis time and peak repeatability, a capillary temperature of 25 °C was selected as the optimal value.

The sample injection time was examined from 3 to 7 s (with the pressure at 2.07 kPa) to enhance the detection limits. The analytes'

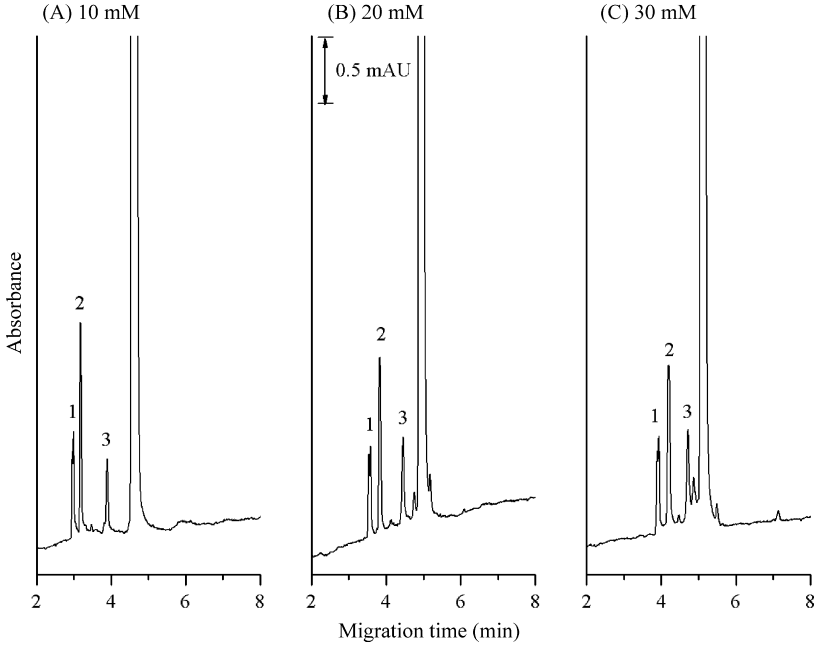

Fig. 5. Electropherograms of the three analytes in BGEs comprising various concentrations of NaOAc (10–30 mM) and 200 mM TFA in MeOH/ACN (10:90, v/v). Other conditions were the same as those used to obtain Fig. 2.

Table 1
Ranges of linearity, calibration curve formulas, coefficients of determination (r^2), limits of detection (LOD), and values of RSDs and theoretical plates for three open-cage fullerenes separated using the optimized NACE method.

	Compound **1**	Compound **2**	Compound **3**
NACE			
Range of linearity (μg/mL)	2.5–35	2.5–35	2.5–35
Calibration curve[a]	$y = 32.58x - 21.03$	$y = 60.05x + 21.10$	$y = 21.43x + 34.46$
Coefficient of determination	0.9930	0.9938	0.9907
LOD (S/N = 3; μg/mL)	0.45	0.27	0.46
LOQ (S/N = 10; μg/mL)	1.49	0.89	1.52
RSD (%; $n = 5$)			
(a) Migration time (min)	0.92	0.85	0.88
(b) Peak height	4.80	5.73	2.75
Theoretical plates (N)[b]	2.92×10^4	7.19×10^4	1.02×10^5

[a] Calibration curve: peak height (arbitrary units) = slope × concentration (μg/mL) + y-intercept.
[b] $N = 5.54(t_R/W_{1/2})^2$; t_R, migration time; $W_{1/2}$, width at half peak height; in 200 mM TFA/20 mM sodium acetate; organic solvent percentage, 10:90 (v/v) MeOH/ACN. Sample concentration, 35 μg/mL.

peak areas increased upon increasing the analytes' injection times. The extended injection times, however, caused insufficient resolution between peaks. To achieve adequate separation efficiency, a sample injection time of 3 s was chosen as the optimized value.

3.6. Method validation

Table 1 lists the ranges of linearity, limits of detection (LODs), theoretical plate numbers (per meter), and relative standard deviations (RSDs) of the migration times and peak heights for the open-cage fullerenes under the optimized NACE conditions. The calibration curve was linear from 2.5 to 35 μg/mL; in this range, the coefficients of determination (r^2) were all greater than 0.9907. The LODs [signal-to-noise (S/N) ratio = 3] of the three analytes ranged from 0.27 to 0.46 ng/mL. In addition, the theoretical plate numbers (per meter), which ranged from 2.92×10^4 to 1.02×10^5, reveal that the peak efficiency was acceptable. Under the optimized separation conditions, the RSDs of the migration times for the three analytes were all less than 1%; for the peak heights, they ranged between 2.75 and 5.73%. Thus, NACE provided high repeatability and separation efficiency for the analysis of these three open-cage fullerenes.

4. Conclusion

We have developed a simple and rapid NACE method, with diode-array detection, for the analysis of three open-cage fullerenes. The nature of the BGE, the organic solvent composition, and the electrolyte concentration all had significant effects on the resolution, selectivity, migration times, and peak shapes. The separation of these three analytes was mediated primarily by the different migration behaviors obtained after protonating their nitrogen atoms, with greater positive charge and smaller size providing more-rapid electrophoretic characteristics. Therefore, under the optimal conditions – 200 mM TFA and 20 mM NaOAc in MeOH/ACN (10:90, v/v) – the three open-cage fullerenes migrated in the order **1** > **2** > **3**. A quantitative analysis of this NACE procedure revealed that (i) the LODs for three compounds ranged from 0.27 to 0.46 μg/mL, (ii) the repeatabilities were adequate, and (iii) the separation efficiency (theoretical plate numbers) was acceptable. Therefore, NACE is a potentially useful approach for analyzing the presence or degrees of purity of open-cage fullerenes.

Acknowledgments

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