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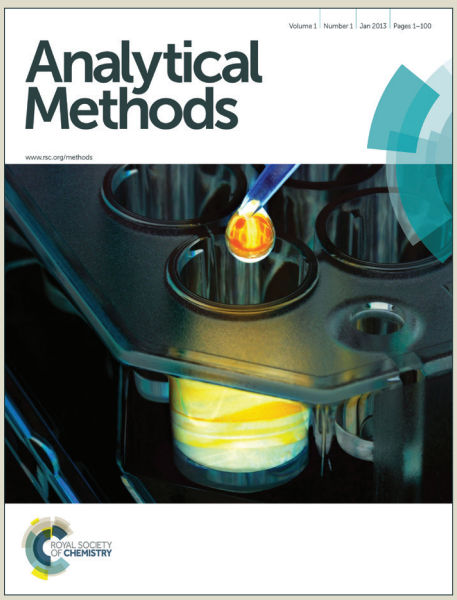

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PAPER

Separation of Carbon Quantum Dots on a C18 Column by a binary gradient elution via HPLC

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Carbon quantum dots (CQDs) have attracted significant attention due to their low toxicity, biocompatibility and potential applications, particularly in the field of biomedical imaging. However, the major drawback limiting the application of CQDs is their relatively low quantum yield (QY). For further study and applications of CQDs, this class of carbon nanomaterials requires separation and purification. In this paper, we report a general method to separate CQDs obtained using resorcinol (*m*-$C_6H_6O_2$) as the carbon precursor on a C18 column through high performance liquid chromatography (HPLC). The separation of CQDs was achieved with a binary gradient elution using acetonitrile/water and acetonitrile/methanol as the mobile phases and acetonitrile plays an important role in the separation of CQDs. The resolution of some peaks was improved when increasing the flow rate; however, the separation of certain other peaks worsened, almost disappearing at higher flow rate. The characterizations of the collected fractions reveal that the oxygen-containing functional groups on the CQDs are crucial when separating the CQDs through this method. Our method is feasible and the collected purified CQDs with QY as high as 0.72.

Introduction

Carbon quantum dots (CQDs) are a unique class of carbon-based nanomaterials that have attracted significant attention due to their unique chemical inertness, biocompatibility and low toxicity. Numerous studies have focused on the synthesis,[1-8] properties,[2-9] and applications [2,3,10-12] of CQDs. Various carbon materials, such as glucose,[4,13,14] resorcinol (*m*-$C_6H_6O_2$),[3] graphene oxide,[10] candle soot[6] and aromatic compounds,[15] have been used as carbon precursors when preparing multicolored CQDs through different methods, such as ultrasonic technologies,[13] solvo-thermal reactions,[10] microwave-assisted technologies,[3-5,14] combustion[6,15] and laser ablation.[1,8] Compared to semiconducting quantum dots, CQDs have been demonstrated low toxicity and good biocompatibility; therefore, they are promising for applications in biomedical imaging.[2,3,10,11]

However, the major drawback limiting the application of CQDs in life sciences is their relatively low quantum yield (QY),[9] we are paying attention to the separation of CQDs. The separation of CQDs samples has been reported previously. CQDs samples derived from candle soot have been separated by denaturing polyacrylamide gel electrophoresis (PAGE), resolving to form three classes of particles.[6] The CQDs prepared through a one-step alkali-assisted electrochemical fabrication are purified and separated into four typically sized CQDs through simple column chromatography.[16] The CQD samples generated through the oxidation of soot are reportedly separated via capillary electrophoresis (CE).[17] CQDs samples synthesized from soot[18] and graphite nanofibers[19] are also separated on a strong anion-exchange column through high performance liquid chromatography (HPLC). Until recently, the separation of fluorescent nitrogen/sulfur-doped carbon dots[20] and CQDs[21] via HPLC coupled with mass spectrometry had just been reported，the elution order of the CQDs species present in the sample follows approximately their core size from small to large.[21] However, the above methods can't get the CQDs with high QYs, an easy and ubiquitous separation method is still necessary for CQDs.

The strong anion-exchange column used in previous studies is a special chromatography column and has a number of limitations during practical usage. Compared to the strong anion-exchange column, reversed phase (RP) C18 columns are commonly used in HPLC with the following advantages. First, the mobile phase for a C18 column mostly consists of a mixture containing water, an organic solvent, and a buffer solution, while the strong anion-exchange column requires a dilute electrolyte solution. Second, a C18 column can be used to separate ionic and nonionic organic compounds, while a strong anion-exchange column is used only to separate ionic organic compounds.

The surface of the CQDs contains numerous oxygen-containing functional groups, such as C=O,[2,4–6,8] C–O–C,[4,5,8] C–OH[4,5] and COO,[7] which may help separate the CQDs. In this study, we developed a general method for separating CQDs obtained using *m*-$C_6H_6O_2$ as the carbon precursor on a RP C18 column with a binary gradient elution via HPLC.

Experimental section

Chemicals

All of the chemicals were analytical grade. Sulfuric acid (H_2SO_4, 96.5%) and *n*-butyl alcohol were purchased from Kelong Technological Co. (Chengdu Sichuan, China). *m*-$C_6H_6O_2$ was purchased from Jinshan Technological Co. (Chengdu Sichuan, China). Quinine sulfate was obtained from Sigma Chemical (USA). HPLC-grade dimethylformamide, dimethylsulfoxide, acetonitrile and methanol were purchased from Tedia (USA). The deionized water (18.2 MΩ/cm) was supplied by a Milli-Q system (Millipore, France).

CQDs synthesis

m-$C_6H_6O_2$ was used to synthesize CQDs through a microwave-assisted method in homogeneous sulfuric acid.[3] Briefly, 1.0000 g of *m*-$C_6H_6O_2$ and 100 μL of H_2SO_4 were dissolved in 2.00 mL of deionized water. Afterward, the mixed solution was heated in a domestic microwave oven (maximum power 800 W, 2450 MHz) for 40 seconds. And then, the volume of the solution decreased and the color changed to wine red. After cooling to room temperature, the product was dissolved in *n*-butyl alcohol, washed until the pH of the washed water was neutral, extracted by rotary evaporator under the vacuum condition at 60 □ and dried. The deep red, viscous liquid was CQDs; the sample was preserved at room temperature. Fifty milligrams of the CQDs were dissolved in 10 mL of methanol, and a 10 times diluent was prepared for separating the CQDs by HPLC.

Characterization

A Techcomp UV1100 UV-visible spectrophotometer (China), a HITACHI F-7000 fluorescence spectrometer (Japan), a Kratos XSAM 800 X-ray photoelectron spectrometer (UK) and a FEI Tecnai F-20 field emission high-resolution transmission electron microscope (USA) were used for preliminary study the properties of the CQDs. The transmission electron microscopy (TEM) and high resolution TEM (HRTEM) images of the CQDs were recorded with a field emission HRTEM (200 kV).

HPLC Analysis

The CQDs were analyzed on a Shimadzu LC-20 system (Japan) equipped with a communications bus module (CBM-20A), pump (LC-20AT), auto-sampler (SIL-20A), column oven (CTO-20AC) and fluorescence detector (RF-10A_{XL}). The C18 column was packed with particles containing 80 Å pores (the Zobax extend-C18 column, 150 mm × 4.6 mm i.d., 5 um, Agilent, USA). The elution conditions involved a gradient of binary mobile phases: solvent A (acetonitrile/methanol, 5/495, v/v) and B (acetonitrile/water, 5/995, v/v). The gradient elution program was as follows: 0 to 5 min 10% solvent A and 90% solvent B; 5 to 8 min solvent A from 10 to 60%, solvent B from 90 to 40%, then holding for two minutes; 10 to 13 min solvent A from 60 to 100%, solvent B from 40% to 0, then holding until 45 min. Before each injection, the chromatographic system should have been equilibrated for at least 15 min. The optimized chromatographic conditions were as follows: the flow rate was 1.4 mL min^{-1}, the temperature was 25 ℃, the excitation wavelength was 320 nm and the emission wavelength was 460 nm.

Results and Discussion

Separation of CQDs

The C18 column exhibited high efficiency and had been commonly used in the laboratory. Numerous conditions were surveyed to separate the CQDs on the C18 column; for example, dimethylformamide and dimethyl sulfoxide were respectively mixed with water to separate the CQDs through isocratic elution methods, the results were disappointing. Acetonitrile/methanol or water/acetonitrile was also used as mobile phases respectively to separate CQDs through isocratic elution, but these conditions were ineffective. Fortunately, a gradient of acetonitrile/methanol and acetonitrile/water could provide good separation for the CQDs. Fig. 1 showed that a few overlapping peaks were eluted quickly during the first few minutes in chromatogram *a* when acetonitrile/methanol was used as mobile phase. No peaks were observed in chromatogram *b* because the mobile phase (acetonitrile/water) had poor elution abilitiy, leaving the CQDs on the column. CQDs were complicated mixture with many oxygen-containing functional groups, neither acetonitrile/methanol nor acetonitrile/water can provide good separation for it. It was reported that the separation of macromolecule required a gradient of the mobile phase with an increasing solvating power in order to achieve the elution of all of the macromolecular components,[22,23] meanwhile one of the solvents must be a thermodynamically good solvent for the macromolecule and the other must be a thermodynamically poor solvent.[24] The sizes of CQDs were less than 10 nm [3-5,8,9] and gradient elution might also been suitable for it. Under the gradient of acetonitrile/methanol and acetonitrile/water, the first peak of CQDs was eluted around 10.00 min when the critical solvent composition[24] was reached (see chromatogram *c* in Fig. 1). As the polarity of mobile phase gradually weakened, many peaks of CQDs were eluted in turn and most peaks had good peak shapes. For instance, peak 3 in 11.12 min had the strongest signal and the largest peak area percentage, peak 14 and 15 almost reached baseline separation. Although 30 peaks were tagged, more peaks could be observed, albeit with weak signal or poor resolution (in the inset of Fig. 1).

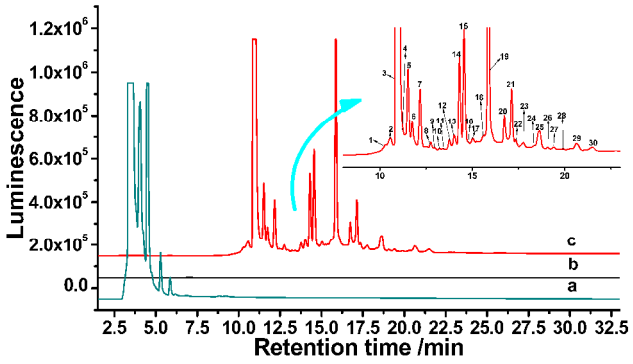

Fig. 1. The separation of CQDs under different mobile phases. The temperature was 25 □, the excitation wavelength was 320 nm and the emission wavelength was 460 nm. The injection volume of the CQDs sample was 0.5 μL (the concentration was 0.5 mg mL^{-1}). An isocratic elution of acetonitrile/methanol at 0.8 mL min^{-1} was used to separate the CQDs in chromatogram *a*. An isocratic elution of acetonitrile/water at 0.8 mL min^{-1} was used to separate the CQDs in chromatogram *b*. Under the optimized chromatographic conditions, a gradient elution of acetonitrile/water and acetonitrile/methanol was used to separate the CQDs in chromatogram *c*, and the inset showed an enlarged portion of chromatogram *c*.

An interesting phenomenon was shown in Supplementary Information Fig. S-1: when methanol and water were used as the mobile phase, the peaks had weak signals and many of the CQDs remained on the C18 column. When acetonitrile/water and acetonitrile/methanol were used as the mobile phase, many peaks had strong signals. For example, the peak intensity of fraction 3 was saturated. Only a small amount of acetonitrile was used in the mobile phase, but it was very importance when separating the CQDs. On the one hand, Acetonitrile was a transitional solvent; on the other hand, methanol, water and acetonitrile adjusted the selectivity of the mobile phase together.[25] Importantly, acetonitrile enhanced the elution ability of mobile phase for the CQDs.

The selected conditions, which involved a gradient with a binary mobile phase, were suitable for separating the CQDs on a C18 column while eliminating or reducing irreversible adsorption.[26,27] Therefore, the separation was highly reproducible (in Fig. S-2 in Supplementary Information).

As shown in fig. 2, the separation of the CQDs was influenced by the flow rate; the faster the flow rate, the better the separation of some of the peaks under the permitted column pressure. The resolution of peaks 14 and 15 (in Supplementary Information Table S-1) increased when increasing the flow rate, and a baseline separation was nearly achieved at 1.4 mL min^{-1}. In addition, peak 4 overlapped with peak 3 completely at 0.6 mL min^{-1} but separated as a shoulder on fraction 3 at 1.4 mL min^{-1}. The greater the flow rate, the worse the separation of other CQDs peaks. For instance, a shoulder peak was observed after peak 20 at 0.6 mL min^{-1}, but it disappeared at 1.4 mL min^{-1} because it overlapped with peak 20. In general, the separation of the CQDs was influenced by the flow rate when flow rate increased from 0.6 to 1.4 mL min^{-1}.

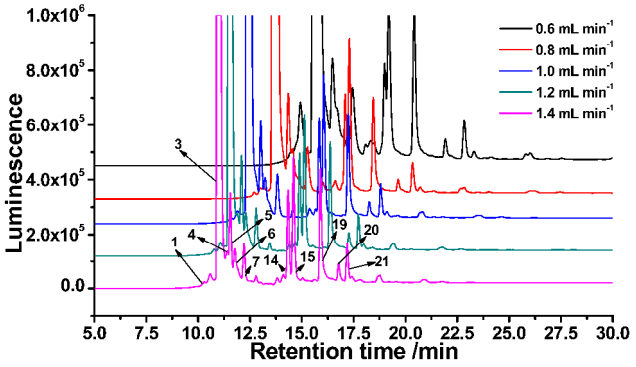

Fig. 2. The separation of CQDs at different flow rates. The flow rate increased from 0.6 to 1.4 mL min^{-1} with an interval of 0.2 mL min^{-1}. The temperature was 25 ℃. The excitation wavelength and emission wavelengths were 320 and 460 nm, respectively. The injection volume of the CQDs samples was 0.5 μL (the concentration was 0.5 mg mL^{-1}). Due to the maximum pressure possible for this chromatographic system, the maximum flow rate was 1.4 mL min^{-1}.

The chromatographic behavior of CQDs was similar to that of other organic compounds; their retention times decreased gradually, and many peaks overlapped when increasing the temperature (see Table S-2 and Fig. S-3 in Supplementary Information). For example, the resolution of fraction 14 and 15 decreased from 1.09 to 0.38 when increasing the temperature; the trend describing changes in the resolution for two peaks was the same as that for the peak shape. Fractions 14 and 15 obviously formed two peaks at 25 ℃, but overlapped to form one peak at 40 ℃. It was reported that the unseparated CQDs had the strongest fluorescence at 460 nm when excited at 330 nm.[3] In our experiment, most peaks of CQDs exhibited strong fluorescence excited at 320 nm when the emission wavelength was 460 nm, as shown in Supplementary Information Fig. S-4. And most of they also exhibited strong fluorescence at the emission wavelength of 460 nm when excated at 320 nm (in Supplementary Information Fig. S-5).

Fraction Collection and Characterization

The properties of the collected fractions of CQDs were interesting during preliminary studies. Under the optimized chromatographic conditions, a solution containing CQDs (5 mg mL^{-1}, 5 μL) was injected over 20 runs. Seventeen fractions were collected and the percentages of the total relative peak area for the collected fractions accounted for more than 90% of the total fluorescence of the CQDs. When using quinine sulfate as a standard,[3,8,28] we estimated the QYs of the seventeen fractions and the unseparated CQDs, the details about the determination of QY were in Supplementary Information. The QYs of the collected fractions were in Supplementary Information Table S-3 and the QY of the unseparated CQDs was 0.42.

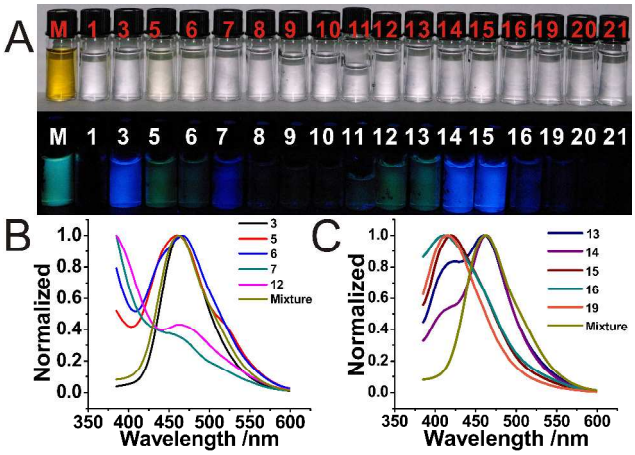

Fig. 3. Optical properties of the CQDs and its fractions under different illuminant-irradiation. The photographs of the unseparated CQDs mixture and seventeen fractions were visualized under white light (top) and under a UV lamp (365 nm, bottom) irradiation in (A). The maximum emission wavelengths of the unseparated CQDs mixture and some fractions were exhibited in (B) and (C) under the excitation wavelength of 320 nm. Ten fractions with obvious fluorescence were mentioned: fraction 3, 5, 6, 7, 12-16 and 19.

As observed in Fig. 3A, the collected fractions and the unseparated CQDs emitted multicolored fluorescence including cyan, blue, indigo, as well as weak fluorescence under a UV lamp (365 nm); meanwhile some fractions exhibited strong fluorescence, while others had weak fluorescence. The unseparated CQDs and ten collected fractions (which emitted cyan, blue and indigo fluorescence) also had different emission wavelengths and intensities at an excitation wavelength of 320 nm when normalized the emission intensity of these fractions (Fig. 3B, 3C). Meanwhile, the fluorescent colour of these fractions corresponded to their maximal emission wavelengths.

As expected, some fractions with strong fluorescence had high QY, while others with weak fluorescence had low QY. For instance, as shown in the inset of Fig.1, fraction 3 had the largest peak intensity and the peak area percentage of which was about 50% of the total fluorescent components of CQDs. And fraction 3 emitted blue fluorescent strongly (Fig.3) with a QY up to 0.76 and the QY of the unseparated CQDs was just 0.42. It fully demonstrated that fraction 3 was one of the main fluorescent components of CQDs and also confirmed that the effectiveness of this method.

The relationship between the oxygen-containing groups on the CQDs and the separation of the CQDs on a C18 column was studied. The XPS spectrum of these CQD fractions was displayed in Supplementary Information Fig. S-6, and six functional groups were present on the CQDs: sp^2C (C=C) at binding energy of 284.5 eV, sp^3C (C-C) at 285.4 eV, C-OH at 286.4 eV, C-O-C at 287.4 eV, C=O at 288.6 eV and –COO at 289.9 eV. In addition, different fractions had different amounts of functional groups (Supplementary Information Table S-3). Previous reports[26,27,29] had pointed that larger nanoparticles were eluted first in size exclusion separations. Recent literature reported that small core clusters were eluted first and larger ones later when separating CQDs via HPLC.[21] Our experiments also confirmed that the mechanism of separating CQDs via HPLC was not a size exclusion process but was instead a reverse-phase chromatographic separation.[27] The separation of CQDs on a C18 column was based on the polarity of CQDs during reversed-phase liquid chromatography, as observed with fraction 3. In Fig. 4, the TEM and HRTEM images showed that the lattice spacing and the average size of fraction 3 were 0.22 nm and 3.0 nm, respectively. The size distribution of fraction 3 was shown in Fig. 4B; the maximum size was 5.0 nm, while the minimum was 1.6 nm with most particles between 2.5-3.5 nm. These particles eluted together when separating the CQDs samples. The characterization results indicated that the different amounts of oxygen-containing functional groups and the large surface-to-volume ratio of the CQDs[1] generated the different polarities, allowing the separation of CQDs. The structures of CQDs obtained from *m*-$C_6H_6O_2$ were similar to graphite,[3] fraction 3 with the lattice spacing of 0.22 nm (Fig. 4C) which corresponded to the (100) facet of graphitic carbon was also confirmed it. It was reported that the size, shape and physico-chemical properties of macromolecules would been changed with the change of environment (e.g. mobile phase composition, flow rate and pressure) and this would change the chromatographic behavior of it, when separating macromolecules by HPLC.[25,27,30,31] CQDs had graphene-like structure, chromatographic behaviors of them were similar to those of macromolecule when separated by HPLC, we tried to explain the chromatographic behaviors of CQDs by the mechanism of separating macromolecule. Specifically, when the column pressure was incereased with the increasing flow rate, the shape of the CQDs might be changed with the increased column pressure, the polarity of the CQDs would been changed due to the uneven distribution of the oxygen-containing groups. Conversely, the shape of the CQDs was changed with the increased column pressure, the polarity of the CQDs remained relatively unchanged when the oxygen-containing groups were distributed evenly.

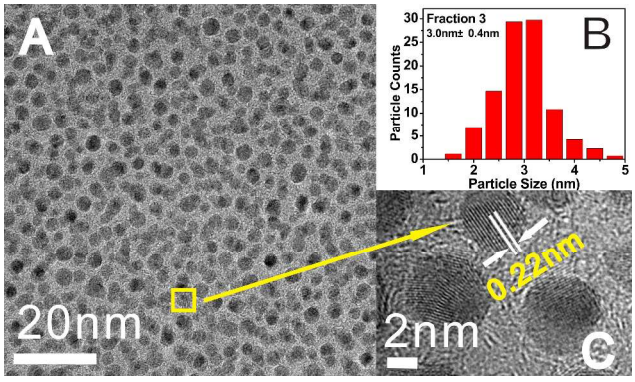

Fig.4. TEM and HRTEM images of fraction 3. (A) TEM image of fraction 3 (20 nm scale bar). (B) Particle size distributions for fraction 3. At least 250 measurements were acquired to measure the particle size distributions. (C) HRTEM image of fraction 3; the crystalline phase of fraction 3 was in the yellow rectangle of image (A) (2 nm scale bar).

Conclusion

In summary, we presented a general and feasible method to separate CQDs on a C18 column via HPLC. The gradient elution of acetonitrile/water and acetonitrile/methanol and the high flow rate were important when separating the CQDs. The resolutions of certain peaks increased, while others decreased when increasing the flow rate under the permitted column pressure. Our study revealed that the oxygen-containing functional groups and the large surface-to-volume ratio of the CQDs generated different polarities, enabling the separation of the CQDs. The collected purified CQDs with high QY might have more applications in the biomedical fields.

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Notes and references

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