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Carbon dots - separative techniques: tools-objective towards green analytical nanometrology focused on bioanalysis

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Abstract

This review aims to focus on the most recent advances and applications of carbon dots (CDs) in the fields of bioanalytical research and clinical analysis involving separation techniques. It includes both facets of the analytical nanoscience and nanotechnology as it is applying analytical nanometrology for CDs and their proper use as tools in (bio)analytical processes. With this purpose, the main properties of carbon dots and the basic synthetic strategies of these materials are summarized, together with the main techniques used for CDs characterization, including the use of separation approaches to describe some of their unique properties. Furthermore, recent and potential further applications and the prospective utilization of these nanomaterials, together with analytical separation techniques, are also discussed.

Keywords: carbon dots, characterization, nanometrology, separation, bioanalysis

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Introduction

Carbon dots (CDs) are nanomaterials that comprise discrete, quasi-spherical nanoparticles with sizes less than 10-nm and an excitation wavelength-dependent luminescent capacity. Currently, they are the low-toxicity and biocompatible alternative to the traditional fluorescent quantum dots [1], first synthesized by 2006, using high energy impact on a carbon target [2,3] or from candle soot [4]. Since then, the scientific community's interest in these materials has grown exponentially, as can be inferred from the number of publications registered in these past years [5] (Figure 1).
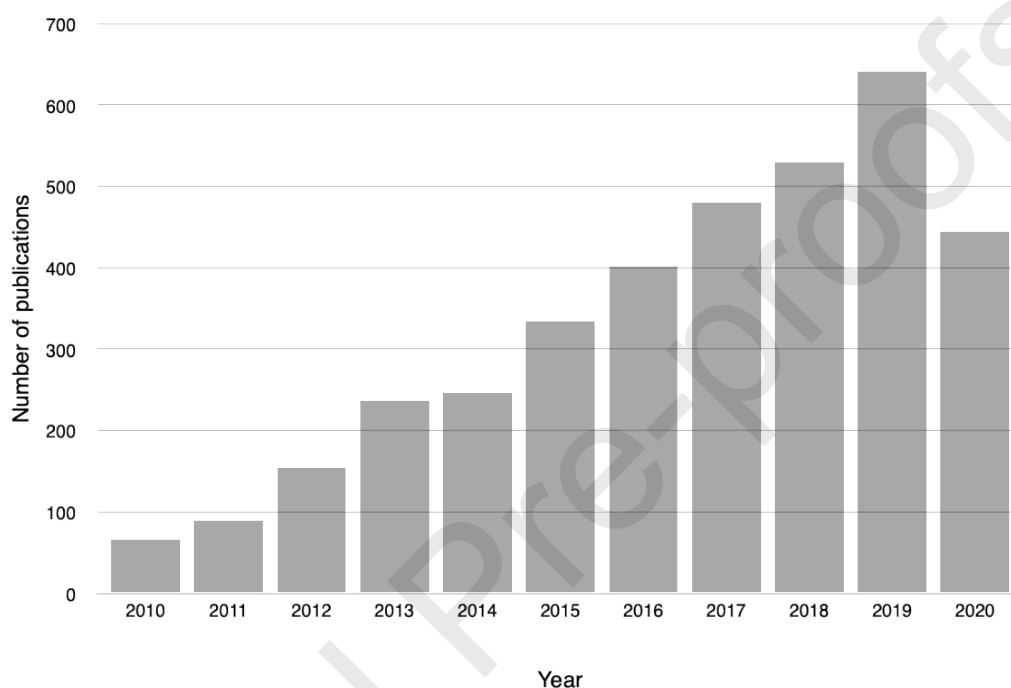

Figure 1. Number of publications, since 2010, including the term "carbon-dot OR graphene quantum dot" in their title. Source: PubMed database search (https://pubmed.ncbi.nlm.nih.gov/).

Several and different approaches have been utilized for their synthesis, either following a procedure comprising an initial two-alternative step of fragmentation (top-down) or the formation from precursors of a carbon source (bottom-up) such as organic solvents, foodstuffs, saccharides, proteins, biomass (e.g., hair) or waste-derived sources [6–8]. CDs prepared by any of these strategies show some similar luminescent properties, though their structures and properties may differ from each other to some extent [9]. Indeed, CDs obtained using the top-down strategy are denoted as graphene quantum dots (GQDs), whereas those synthesized by the bottom-up approach are named as carbon nanodots [10]. Nevertheless, the term carbon dot will be applied in this revision to any of them, regardless of their synthesis approach.

Once the carbon crystallized core is formed, a surface modification stage of passivation or functionalization is performed to acquire fluorescent properties. Different physical or chemical methods have been successfully applied for CDs synthesis. Nevertheless, this work will not focus on this regard as extensive reviews regarding this issue have been previously published [5,9,11,12]. No need for a complex, time-consuming, inefficient, or expensive synthesis process is required to fabricate these nanoparticles. More interestingly, green synthesis approaches are considered as of the abundance of natural sources of precursors, the decrease of exposure to

different chemicals, and the rate of waste generation, thus allowing an economical and renewal process of synthesis, even though it still lacks relevant development [13].

With regard to the surface modification of CDs, different types of functional groups are susceptible of being added to the carbon core: organic polymers (e.g. polyethylene-glycol (PEG) [3,14,15], propionylethylenimine-co-ethylenimine (PPEI-EI) [2,3]), or inorganic elements such as the N-doped [16], ZnO/ZnS [15], or TiO_2/SiO_2 [17] CDs. However, it is still controversial the main origin of the photoluminescence ability of CDs, especially for their up-conversion capacity as not only the surface passivation of the particles during CDs synthesis or later is related to this phenomenon, but also the size-dependent quantum effect is thought to be responsible for the particular optical features of CDs [1,9]. Additionally, CDs possess high aqueous solubility due to the presence of carboxylic acid moieties at their surface, chemical inertness, and lack of optical blinking and show higher resistance to photobleaching than traditional semiconductor quantum dots and organic dyes. Their magnetic, optical, and electronic features, together with their biocompatibility and low cytotoxicity, make CDs a highly valuable tool to be applied in bioanalysis, bioimaging, drug delivery, and as biosensors [18,19].

Moreover, analytical techniques, materials, and approaches are taking advantage of this type of nanomaterial. In this review, we aim to focus on the most recent advances and applications of CDs in the fields of bioanalytical research and clinical analysis utilizing separation techniques and methodologies. With this purpose, the main properties of CDs and the basic synthetic strategies of these materials are resumed, together with the main techniques used for CDs characterization. According to their analytical features, the use of separation approaches to describe some of the unique properties of these nanomaterials is also discussed. Finally, recent and potential further applications, and the prospective utilization of these nanomaterials and analytical separation techniques are also analysed.

Techniques for CDs characterization

Nanometrology is the field of science that aims to standardize physical measurements at the nanometre scale. In this case, apart from their physical properties (i.e. metrics), CDs can be described by their chemical composition. The particular electronic, magnetic and optical properties of carbon nanomaterials are mainly based on their structural diversity, which is essential for their adequate characterization and comprehensive analysis of their properties and, consequently, to predict their potential applications in biomedical and bioanalytical fields. CDs are quasi-spherical carbon nanoparticles of diameter lower than 10 nm and with high oxygen content due to the presence of carbonyl, carboxyl or epoxy groups [9,11], as a result of combining different volumetric ratios of graphitic and turbostratic carbon [20]. Thus, several experimental techniques are required to obtain not only the information on the crystalline organization of the carbon atoms within the nuclear core of CDs but also to perform a qualitative and quantitative analysis of the functional motifs potentially present in the surface of CDs. Different approaches are therefore required to elucidate essential features of CDs such as their structure and composition: microscopy, spectroscopy and elemental analysis [21,22].

Transmission electron microscopy (TEM) and X-ray diffraction (XRD) represent essential techniques for elucidating the morphology, crystalline organization, and size distribution of these nanomaterials. In this sense, high-resolution TEM (HRTEM) experiments can confirm the periodicity of the graphitic core indicative of the crystalline or amorphous nature of CDs, as well as their images, reveal the degree of dispersion of these

nanoparticles and whether a narrow or wide size distribution, in terms of particle's diameter, is detected [23] (Figure 2). Almost every approach for C-dots synthesis is characterized in terms of particle size distribution, morphology, lattice planes and crystalline organization, and confirmed by HRTEM experiments [24]. XRD adds information upon the unit cell dimensions and crystal spacing within the crystalline carbon cores, representing necessary structural data on the crystalline nature of CDs [9], and ^{13}C NMR spectroscopy is a powerful technique in distinguishing sp^3 carbons from the sp^2 ones. It allows us to gain further structural insights about carbon nanoparticles. ^{13}C NMR measurements reveal such structural details, as aliphatic (sp^3) and aromatic (sp^2) carbons resonate differently, with the former in the range of 8 to 80 ppm whereas the latter is 90 to 180 ppm [25].
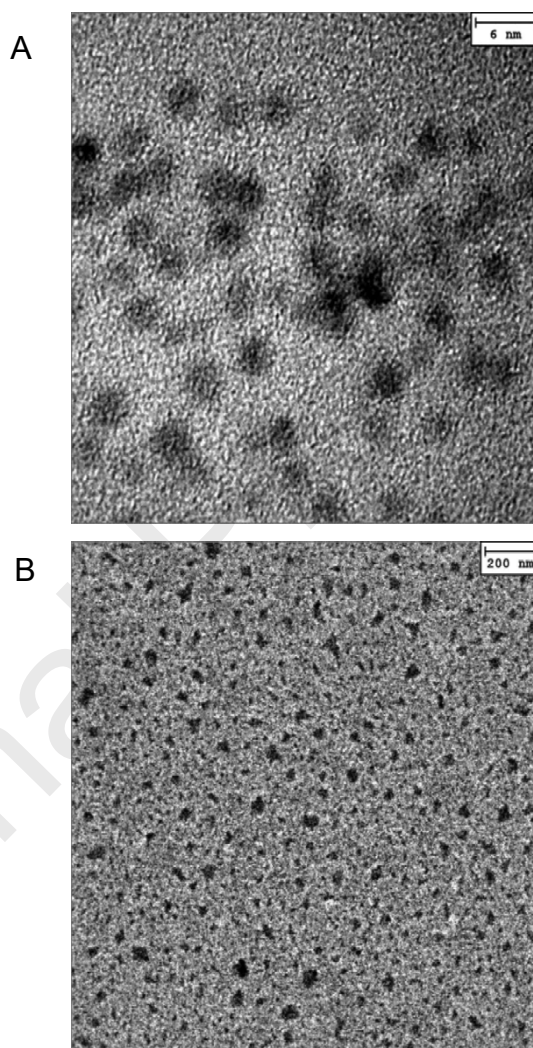

Figure 2. TEM images of (A) CDs and (B) N-CDs nanoparticles obtained from aqueous ammonia aqueous solution [23]. Reproduced with the permission of Elsevier B.V.

Atomic force microscopy (AFM) and scanning tunnelling microscopy (STM) are additionally applied to determine the size of CDs and surface morphologies to the resolution of a fraction of nanometres. In AFM, the atoms of a cantilever tip and sample surface exert force on each other, which is measured for the different heights of the sample surface during the scan, thus creating a height/position profile of the CDs regarding the substrate. A minimum change in the height of the cantilever tip deflects laser, registered by a photodiode, amplified and coded to create an image of the sample [22]. AFM images can therefore reveal the products' range of diameters,

the overall dot size [26], the morphology and sectional analysis [27], as well as patterns of distribution of CDs upon a surface. AFM can overestimate the width of the CDs due to the AFM probe interaction with the features, though their height can be extracted from the AFM images [28,29].

Similarly, STM requires a continuous voltage between the STM probes, thus creating tunnelling of electrons between atoms of the sample surface once brought very close to each other. These electrons, therefore, create a change in the current probe, and the rate of current change to height is used to form the STM image. For instance, Nguyen et al. utilized a solvothermal synthesis route with further purification and single-particle characterization of the electronic structure and optical absorption of CDs. This would prevent signal contaminations from impurities usually detected in optical spectroscopy. It was specifically applied a single-molecule absorption detected by scanning tunnelling microscopy to image the excited state of CDs under laser illumination, and the images showed that the graphitic core possess a larger bandgap consistent with fluorescence, whereas the optically active surface defects show electronic gaps consistent with the emission wavelength of CDs. This observation supported energy transfer from the graphitic core to highly localized surface defects for their CDs [30].

Moreover, Raman spectroscopy may reveal the structural features of the carbon atoms within CDs. Two broad peaks, at around 1300 cm^{-1} and 1580 cm^{-1}, corresponding to the D (disordered sp^3 carbons) and G bands, respectively, are typically observed within a Raman spectrum of CDs. The crystalline G band is related to the in-plane vibration of the sp^2 carbon. The relative intensity (I_D/I_G) of these bands are useful to determine the structural properties of the carbon framework, particularly the degree of crystallinity and relative abundance for the inner of CDs [31].

Dynamic light scattering (DLS) measures the size and size distribution of molecules and particles, typically in the range of a few nanometres. The Brownian motion of particles (molecules) in solution causes a scattering of the laser light at different intensities, and the analysis of these intensity fluctuations will yield the velocity of the Brownian motion and, thus, the particle size using the Stokes-Einstein relationship. On the other hand, the elemental composition and surface functional groups of CDs can be carried out and confirmed by X-Ray Photoelectron Spectroscopy (XPS) and Fourier Transform Infrared (FTIR). During CDs synthesis, different metal and non-metal-containing functional groups (e.g. hydroxyl, carboxyl, ether, epoxy, amine, sulphur, silicon, boron, or phosphorous) are attached to the surface of CDs by passivation, thus allowing CDs to play a crucial role in each particular application. Indeed, metal atom doping can intimately modulate the band structure of the CDs, thus modifying not only the optical properties but also creating functionalities for these so-doped CDs. XPS provides information upon specific atomic units present upon CDs' surface by analysing their spectra analysis (Figure 3).

On the other hand, FTIR, based on the principle of Michelson interferometer, complements XPS or determines the cause of the optical properties of CDs by observing the typical vibration bands within the spectral window of 500–4000 cm^{-1}. Any change in FTIR spectra can be useful to confirm chemical interactions (e.g. weak hydrogen bonds or strong amide linkages) [32]. Additionally, it has been recently suggested that the electronic, optical and catalytic properties of CDs not only depend on the functional surface groups, but they are also related to doping of foreign atoms into the cores. Unfortunately, the techniques required for the deconvolution

of the fine core–surface (interior and surface functional groups) structures of CDs remain limited, thus requiring further research [21].
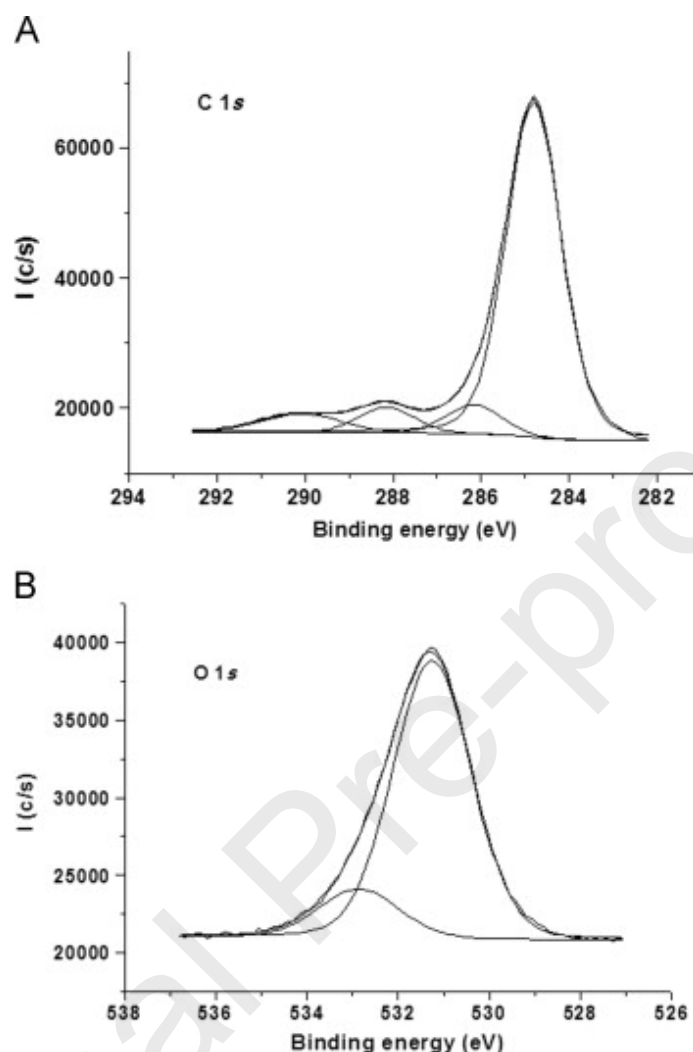

Figure 3. XPS spectra of the binding energies obtained for the C 1s (A) and (B) O 1s cores level constituting CDs [33]. Reproduced with permission of Elsevier B.V.

Photoluminescence properties

CDs show interesting optical features, particularly as of their excitation dependent emission spectra, which gives rise to fluorescence, as well as high fluorescence quantum yields, with reported values up to 94%, long photoluminescence (PL) decay lifetimes, and photostability [34–38]. Additionally, they possess particular optical properties that may reflect impacts from particles of various sizes in the sample, as well as different emissive sites are distributed on each carbon dot [39]. Nevertheless, the mechanism of PL is not well understood yet, but fluorescent carbon nanoparticles with tuneable emission would be considered as next generation green nanomaterials and an alternative to fluorescent semiconductor nanocrystals, usually comprising toxic heavy metals (e.g. cadmium). The following techniques are used for optical and photoluminescence analysis of CDs.

UV spectroscopy

The excitation wavelengths and the absorbance spectra of CDs are determined utilizing UV-Vis spectroscopy. Carbon dots show strong absorption in the UV region (approximately in the 200-320 nm interval), though lower

intensity of absorption within the visible region. Either π-electrons or non-bonding electrons (n-electrons) of the molecules can absorb the energy of radiation depending upon energy gap up to higher anti-bonding molecular orbitals to excite (e.g. a 260–320 nm band is known as a π–π* transition peak in most CDs). Likewise, a shoulder peak in the 270–390 nm range, possibly attributable to the n–π* transition of C=O bonds is observed for graphene QDs [40].

The positions of CDs absorption peaks of UV are different according to the synthesis approach utilized, the size and the surface functional groups [39,41]. Interestingly, the absorption wavelength may increase with the emission wavelength red shift after doping and surface passivation, which is a common phenomenon for multi-emission CDs [31]. In this sense, CDs with long-wavelength and multicolour emission could be prepared by selecting the adequate materials for their synthesis [31]. At the same time, surface functionalization not only is a prerequisite for further applications of CDs, but this process also significantly influences their PL properties, by stabilizing fluorescence or improving the fluorescence quantum yields [5]. With this purpose, surface modification, element doping and creating composites or hybrids are effective approaches to attain the functionalization of CDs [39,41,42].

Fluorescence Spectrometry

The optical behaviour of CDs may be a consequence, not only from the existence of particles of different sizes in the sample but also from the distribution of different emissive sites on each CD. In this sense, fluorescence spectrometry helps to determine the size and concentration of CDs according to their fluorescence properties.

Luminescence decays showed that CDs produced by many methods have multiexponential PL decays [43], thus indicating the presence of different emissive sites. The synergistic effect of the carbon core and the surface/molecule state contributed to the PL of CDs. Interestingly, some studies have shown the reversibility of the fluorescence process for CDs: after fluorescence quenching of CDs by acidification, subsequent re-esterification reversed the process, a phenomenon which is probably caused by changes in the surface energy states directly affected by this chemical reaction [44]. Nevertheless, these nanomaterials still suffer from low photoluminescence quantum yields in the red part of the spectrum, and further research is warranted to how effectively realize the long-wavelength and multicolour emission through appropriate synthesis, even though some progress has been made [45–48]. Consequently, a full description of the photoluminescence properties of CDs requires both UV-Vis and fluorescence spectroscopy.

C-dots: target analyte of separation techniques

Separative approaches have played a key role in CDs characterisation since its discovery from their isolation from single-walled carbon nanotubes by electrophoresis [49]. More indeed, a post-synthetic strategy implementation serves to obtain purified and mono-disperse CDs that are independent (e.g. in size, emission). Likewise, surface functionalization occurs during the synthesis procedure or post-synthesis, which is of great significance to the properties and applications of CDs. All those processes require of techniques of different complexity, ranging from simple and complementary treatments such as rotatory evaporation [50], centrifugation, dialysis [51] and filtration, to more precise and complex techniques (i.e. electrophoresis, silica column chromatography [52], or high-performance liquid chromatography (HPLC) [31]), to enhance our knowledge on the chemical and photophysical properties of high-purity CDs fractions.

Electrophoresis

Gel electrophoresis and capillary electrophoresis (CE) have both emerged as a powerful approach for high-efficient separation of nanomaterials (e.g. nanoparticles). However, not much work applying this technique has been used for analysing the effect of synthetic conditions on the formation and property of CDs product.

Preparative gel electrophoresis, together with a subsequent PVDF filtration and dialysis for further purification, was applied when CDs were accidentally found at the purification process of SWCNT from arc-discharged soot. A 1% agarose gel slab was utilized, and two bands of different nanomaterial were obtained: short tubular carbon and green-blue, yellow, and orange fluorescent carbon nanoparticles [49]. Likewise, the interaction between CDs and tyrosinase to generate a hybrid fluorescent probe for the detection of dopamine was explored by mixing these components in solution and then separating the resulting hybrids by sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Thus, it could be determined that CDs and tyrosinase formed stable complexes and, under UV light irradiation, CDs were observed within the complexes, as well as they were found to be more mobile than the formed hybrids, which was explained as of a higher the hydrodynamic radius of the CDs upon adsorption of the PPL. Additionally, it could be observed the non-covalent bonding of CDs to the tyrosinase [53].

Similarly, the surface functionality of CDs can be tailored using different passivating agents. In this sense, the optical performance and bioimaging competence of different surface functionalized CDs with polyethylene glycol (PEG) or polyethyleneimine (PEI) was evaluated, to analyse their potential as biocompatible imaging agents using normal (BHK-21) and cancer cell lines (A549). The agarose gel assay, the SDS-PAGE analysis and zeta potential confirmed that CD-PEI is positively charged as of the presence of amine groups on its surface and, therefore, confirming its ability to bind to the cell membrane through electrostatic interactions, in contrast to CD-PEG, which possess a negatively charged surface [54]. However, SDS-PAGE is not highly efficient regarding its capacity to separate molecules, even though its remarkable capacity to identify the relationship between the mobility and colour of CDs. Electrophoresis is additionally used for product purification of CDs synthesized on a different way, by oxidative acid treatment of candle soot, to form hydrophilic CDs by decomposing carbon aggregates into small size nanoparticles. The CDs were then purified using polyacrylamide gel electrophoresis (PAGE), which in turn enhanced their luminescent properties [4].

On the other hand, capillary zone electrophoresis (CZE) is an efficient separation technique, with large peak capacity and a remarkably low amount of sample required. Nevertheless, the influence of the electrophoretic parameters on the sample separation needs to be well understood for its proper application in CDs samples' fractionation for its analysis. Baker et al. used CE-UV approach for the analysis of carbon nanoparticles mixture generated by oxidation of soot from the flame of an oil lamp. They studied the role of buffer composition, counter-ion, pH and ionic strength on the electrophoretic mobilities of this sample. Then, they concluded the occurrence of the dependence of mobility on the buffer ionic strength thus reinforcing the theory of the existence of highly charged particles within the nanometre size range, as well as the influence of buffer pH on the electrophoretic pattern [55].

The complexity of CDs synthesized from citric acid (CA) and 1,2-ethylenediamine (EDA) by microwave-assisted pyrolysis under controlled pH, reaction time and the ratio of amine to carboxylic acid, was checked using CZE-

UV and laser-induced fluorescence spectroscopy. A 40 cm capillary (50 μm i.d. and 325 μm o.d.) with 30mM sodium acetate-acetic acid at pH 3.6 as run buffer and an applied voltage of 15 kV were utilised. This technique ensured good levels of sensitivity and accuracy for these CDs analysis. Given the fact that the CDs produced in this work comprised amine, amide and carboxylic acid moieties, CDs were present as different ions in aqueous solution, thus making CZE an optimal approach for the analysis of their electrophoretic behaviour, and helping to optimize the conditions for the synthesis of specific CDs of particular composition [56].

CZE-UV at 250 nm and laser-induced fluorescence detection at λ_{ex} and λ_{em} of 488 and 550 nm, respectively, was also utilized by Liu et al. to study the reaction time-associated kinetics of CDs formation from glacial acetic acid and diphosphorus pentoxide without external heating, and to identify the functional groups-associated charge states successfully. CDs were separated with a 40 cm capillary (50 μm i.d. and 325 μm o.d.), a running buffer composed by 10mM SDS and 30mM phosphate at pH 9.0 and applying 15 kV of voltage. Unfortunately, the low volume of sample used requires an excessive time to collect enough amounts of CDs fractions, as well as only charged CDs species could be separated, while neutral CDs eluted joint as a single peak. Nevertheless, it could be observed the existent differences in absorption, emission characteristics, and PL quantum yield of the fractionated species [57]. CE with fluorescence detection is also utilized to characterize specific properties of CDs, such as the pK_a value of nitrogen-doped GQDs (N-GQDs) prepared by a facile solvothermal treatment of graphene oxide using dimethylformamide, which was used for Al^{3+} detection in aqueous media and living cells. This pK_a value helped to shed light on the pH-dependent fluorescence enhancing the efficiency of N-GQD induced by Al^{3+}. More specifically, the solute fully ionized showed the maximum electrophoretic mobility, while being the lowest when protonated and had intermediate mobility under pH values surrounding its pK_a. Values of pH ranging from 3.0 to 10.0 did not change the fluorescence intensity of N-GQD, contrarily to the increased fluorescence of N-GQD by Al^{3+}, which was significantly affected, being pH 4 the value at which the highest enhancing efficiency occurred [58].

To fully explore the potential of CDs can work as biocompatible fluorescent labels when they are linked to biomolecules (e.g. antibodies and nucleic acids -aptamers-) through bifunctional linkers, or homofunctional linkages such as glutaraldehyde, and electrostatic attraction, efficient separation of conjugates from free CDs and biorecognition units is highly beneficial. Wu et al. described a CE-UV method for both the quantitation of CDs and to analyse the CD-antibody bioconjugate to obtain the desired CD bioconjugate. A bare silica capillary with an effective length of 38.5 cm and an inner diameter of 74 μm, and 100 mM tris-acetate at 8.4 pH as a working buffer, was utilized. A positive voltage of 20 kV was applied for the electrophoretic separation of the C-dots, while 12 kV was applied to separate CDs and bioconjugates. The amine functional groups of the CDs were linked with the bioactive proteins through a homofunctional crosslinker, glutaraldehyde, to create the biolabeling. The CD labelled anti-AFP was then analysed by CE, and the conjugated CD-anti-AFP was more negatively charged than the unlabelled anti-AFP arising from the attached, negatively charged C-dots. The increased negative charge on the conjugate was possibly the main causative factor in the shift of migration to a longer time in this case, because the attached C-dots, being small relative to the conjugate, made the size difference between CD-anti-AFP and anti-AFP a minor influencing factor in determining migration rate. However, compared to the un-functionalized C-dots, CD-anti-AFP conjugates have a much larger size. CE was, therefore, applied to the optimisation of CD labelling of antibodies [59]. It is noteworthy that the calibration curve

required adjustment when CDs are synthesized from other sources and other preparation methods, thus remarking the importance of the existence of certified reference materials. Likewise, a nanosystem for cancer cell-targeted imaging, composed by DNA aptamer AS1411 modified CDs with polyethyleneimine (PEI) as the connecting bridge, was synthesized and CDs, CDs-PEI and CDs-PEI-AS1411 further characterized using CE by Kong et al. [60].

Chromatography

C-dots exhibiting diverse luminescence properties are obtained after fractionation of CDs sample, thus aiding to determine parameters and mechanisms responsible for CDs excitation-dependent emission, as well as the purification of CDs samples into discrete sizes and/or shapes adds consistency for biological applications and facilitates their translation into clinical uses. With this purpose, up to date, different modes of chromatography have been utilized: silica gel column [61–63], size exclusion chromatography (SEC) [64], ion-exchange [65], normal or reverse phase chromatography [66–68]. Compared with the electrophoretic separation techniques, higher peak resolution together with more information about spectral and chemical properties of the individual separated CDs can be obtained due to its potential coupling with different modes of detection, UV, fluorescence detection or mass spectrometry, respectively.

Mn(II)-coordinated CDs prepared by solvothermal treatment of p-phenylenediamine and $MnCl_2 \cdot 4H_2O$ were separated by silica column chromatography using petroleum ether:ethyl acetate as the eluent, obtaining four fractions showing different fluorescence characteristics, though none exhibited excitation-dependent fluorescence [52]. Size exclusion chromatography (SEC) allowed isolating the most luminescent fraction of a sample of CDs synthesized from citric acid monohydrate and urea by microwave irradiation, previously concentrated to about 4 g L^{-1} by rotatory evaporation [50]. The separation was performed using a stationary phase consisted of three resins with increasing cut-off values, selected according to the correlation between the hydrodynamic radius and the average molecular weight of random polymers previously observed. Consequently, resins were adequate to separate CDs in the 1−6 nm range of diameters. Ultrapure water was added on the top as eluant, and four different fractions of CDs with distinct morphological and optical characteristics were identified [50]. SEC purification can be versatile about to the medium type, column, particle and pore sizes, and solvent, thus allowing its application for various types of CDs [64]. However, a long time is required to performed such kind of fractionations. In this sense, some slightly faster separations of GQDs have been performed by Fuyuno et al., who separated GQDs used fabricated by chemical oxidation of pitch-based carbon fibres in 46 min. Fractions were collected every 15 minutes, using three columns connected (Cosmosil CNT-2000, CNT-1000, and CNT-300), with different pore sizes of 2000, 1000, and 300 Å, and utilizing phosphate buffer (pH 7.0) containing sodium sulphate for optical measurement samples and milli-Q water for the TEM and XPS measurements as the mobile phase [69]. In all these cases, as expected by the principle of size-exclusion chromatography, the overall size of the GQDs decreases with increasing HPLC retention time.

Likewise, ion exchange-HPLC is a separation technique which allows the separation of charged ions or molecules depending on their affinity to the ion exchange resin containing charged groups. For instance, Vinci et al. applied a semipreparative anion-exchange HPLC to CDs mixtures produced from graphite nanofibers to obtain fractions with refined and unique properties not observed at the unseparated mixture (Figure 4). The isolated fractions were collected based on the laser-induced photoluminescence detection and subsequently

analysed by CE, thus confirming the presence of one predominant species at each fraction collected, with remarkable charge/size homogeneity. Generated fractions of CDs possessed harper emission spectra and greater quantum yields and brightness, compared to the "parent" CDs [65]. It was also reported that the fractions collected produced excitation-independent fluorescence emission, contrarily to the excitation-dependent emission spectra obtained by the initial sample, which is concordant with the theory of the existence of excitation-dependent emission phenomena due to the presence of many fluorophores (each exhibiting distinct structural features) in a sample [5]. In an attempt of analysing the surface properties of the isolated fractions of the graphite nanofiber-derived C-dots with relatively high photoluminescence, this same group reported the fractionation of CDs mix by AE-HPLC on a semi-preparative column (9.0 mm inner diameter × 250 mm length) coupled with UV detection at 250 nm and with laser-induced photoluminescence (LIP). A flow rate of 5 mL/min and gradient elution from 100 mM to 300 mM ammonium carbonate salt (pH 9.0) was carried out for the separation of 11 peaks identified based on their LIP signal [70].

Reverse phase (RP)-HPLC is based on the use of a mobile phase with higher polarity than that of the stationary phase, an adequate technique to separate non-volatile and thermolabile compounds. Additionally, it allows to be used at the preparative scale thus potentially collecting higher amounts at individual fractions, and neutral CDs can be efficiently retained and separated, which, in turn, keep together when using CZE. First, Hu et al. attained a high-resolution separation of CDs prepared by hydrothermal carbonization of chitosan by using an RP-HPLC method coupled with both UV and fluorescence detection. C_{18} column, with methanol and Milli-Q water as the mobile phase, was applied to separate the initial sample in 12 separated CDs fractions, which were subsequently analysed by UV-Vis, PL spectroscopy and TEM [67]. More indeed, to study the surface-attached functionalities of these fractions, MALDI-TOF-MS was applied to analyse their fragmentation patterns, even though this technique cannot assign the exact highest mass ions of each CDs fractions. Likewise, this group also utilized RP-HPLC coupled with fluorescence or UV detection to fractionate and analyse CDs and hollow CDs samples, respectively, using ammonium acetate and methanol as mobile phase [66,68]. In this sense, the chromatograms obtained showed that a lower organic solvent content tends to increase the retention of the CDs species can be increased if lower proportion of organic solvents is used as better hydrophobic interaction between the CDs and C_{18} column, and vice versa. Additionally, it is also expected that only neutral and not charged CDs will retain on this stationary phase [66]. They obtained multicolour emissive fractions, able to display red, green, and blue fluorescence under UV irradiation (365 nm), which were then used for living cell imaging after checking their biocompatibility.
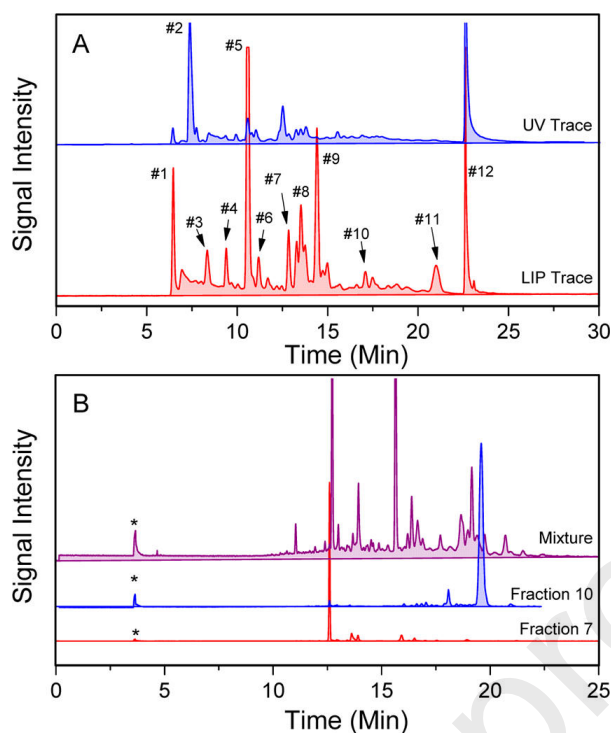
Figure 4. (A) AE-HPLC chromatograms of a 1−50 kDa mixture of CNPs monitored by UV absorption at 250 nm and LIP (λex = 325 nm, luminescence collected through 350 nm long pass filter). (B) Electropherograms of the CNP mix and of HPLC-collected fractions 7 and 10, monitored via LIP (λex = 488 nm, luminescence collected through a 520 nm band-pass filter). The electroosmotic flow (EOF) marker is identified with an asterisk [65]. Reproduced with permission of the American Chemical Society.

More recently, ultra-performance liquid chromatography (UPLC) coupled with either UV detection [71] or MS [72,73] detection has been successfully employed to separate and analyse CDs species [73]. UPLC accelerates the separation and fractionation step, enhances the separation efficiency, sample loading, and solvent consumption, as well as it can be coupled with tandem mass spectrometry (MS/MS) for the soft ionization and sensitive MS detection of each species obtained, as mass spectrometry reveals information on the chemical structures of smaller-sized nanomaterials (< 5 nm). An interesting example is the application of UPLC coupled with ESI-Q-TOF-MS/MS to separate and then characterize CDs synthesized from citric acid and 1,2-ethylenediamine by microwave-assisted pyrolysis [72]. CDs species were adequately separated within 4.0 min. In contrast, the simultaneous use of ESI-Q-TOF-MS/MS helped to the characterization of the chemical structures and the elucidation of their molecular formulas, revealing, for the first time, that CDs exist as individual monomers linked through non-covalent bonding forces to form supramolecular structures [72]. Likewise, Gong et al. employed UHPLC and HPLC to separate, fractionate and characterize CDs samples prepared to apply microwave energy to carbonize chitosan and acetic acid under various synthesis conditions of microwave time, quantities of acetic acid and chitosan [71]. However, the collected fractions after UHPLC are too reduced for further characterization, so HPLC is then utilized to collect CDs fractions for subsequent studies of photoluminescence MALDI-TOF MS. The conditions for UHPLC are slightly different from those of HPLC, as a smaller column is utilized (2.1 mm i.d. x 150 mm, 1.7 μm of particle size), and more reduced volume of sample is injected (1.0 μL). Unfortunately, the highest mass ions of CDs fractions could not be accurately assigned by

MALDI-TOF MS, though it elucidated the surface-attached functionalities through the analysis of the observed fragmentation patterns [71].

Even though the sound advantages of using chromatographic techniques for isolating CDs species from complex mixtures, this technique is uniquely reported in less than 10% of the published purification protocols for carbon dots [64]. Nevertheless, a scalable, analytically efficient separation approach is still required to obtain high-purity, single-component carbon dot samples in quantities over those obtained at lab synthesis scale, thus being a remarkable challenge to attain for researchers interested on CDs nature and applications [64].

Extraction techniques

Amongst these separation processes used to produce post-synthesis uniform CDs, high-speed centrifugation techniques can be utilized [74]. Moreover, cascade sedimentation as a function on their mass is effective for size fractionation, though limited when small differences in mass exist [75]. The density gradient centrifugation method, usually applied to separate biomacromolecules, can be successfully utilized to obtain CDs. Briefly, samples are put on the top of a density gradient formed by sequentially layering solutions of different densities. According to their sizes, shapes, or densities, the different particles will deposit in the density gradient at different speeds on the basis when centrifugation is applied. As C-dots are usually synthesized in aqueous medium and possesses excellent water solubility, so no issues related to coagulation phenomena, due to changes in solvent nature, occur. For instance, sucrose density gradient centrifugation was carried out, as a non-toxic alternative, to separate CDs from other carbon-based materials [76]. First, 100% pure sucrose was poured at the bottom of a tube, and then 50% sucrose just over it. 2 ml of the sample was then situated on the top and spun for 30 minutes at 6000 rpm. This sharp density gradient allows fine size-based separations [77]. Sahu et al. for the first time reported the use of differential centrifugation to separate highly fluorescent almost monodisperse (CD) and less fluorescent coarse particles. The aqueous solution containing CDs was firstly centrifuged at 3000 rpm for 15 min to get less-fluorescent deposit coarse nanoparticles of 30-50 nm size. Then, to the upper brown solution, an excess amount of acetone was added and centrifuged at 10000 rpm for 15 min: the supernatant contained the CDs of 1.5–4.5 nm averaged size [78].

Additionally, solute sizes with selective cut-off can be selected by filtration through a membrane, according to the pore size [79–81]. In this sense, pressure-driven membrane filtration, under two different modes (i.e. dead-end and cross-flow filtration [82]) are applied. It, for example, has been used for large scale preparation of graphene quantum dots, and their separation from larger-sized particles, by filtration through a 100-nm microporous membrane, its subsequent centrifugation at 10000 rpm for 1h and a final dialysis step using a dialysis bag, with a cut-off molecular weight of 103 Da for 72h [83]. More specifically, in dead-end filtration, the feed flow is subjected to parallel permeation (filtrated) flow and the feed solution completely passes through the membrane, similarly to filtration using a syringe. However, particles larger than the pore size may accumulate on the membrane surface or remain plugged within the membrane, and surface cleaning of the membrane or membrane replacement is required to recover the permeation rate [79]. Moreover, fouling on the membrane surface may occur when filtrating CDs solutions, so an additional treatment to ensure high purity is required [82,83].

Alternatively, in cross-flow filtration, the initial sample passes tangentially along the surface of the membrane. A pressure difference across membrane drives through the membrane those components smaller than the pores, whereas larger elements are swept along the membrane surface with the cross-flow of the fluid [79]. Therefore, these particles will flow back to the sample reservoir, thus reducing membrane fouling, providing a more stable flux, better permeation rates and increase durability of filters [84]. Cross-flow velocity mainly determines mass transfer through each given membrane and sample solution [85]. Nevertheless, this approach has been scarcely applied for fractionating or purification of 2D nanomaterials, such as GQDs or CDs [79,86,87]. For instance, Yim et al. recently developed a method for an effective fractionation, by size, of GQDs dispersed in water using cross-flow filtration with uniform pore size membranes. Selective permeation of relatively small GQDs through the membrane occurred for a given pair of cross-flow velocity and pore size values, with higher selectivity and permeability than using a dead-end filtration system [79]. Nevertheless, the molecular weight cut-off of the membranes, as well as the step-by-step process (in terms of time and water replacement), requires of optimization for each specific nanomaterial, as recently reported and suggested [64]. Similarly to the solvent extraction process, it is not capable of efficiently separating each CDs species in some instances due to the complexity of some CDs synthesized, which are multicomponent systems.

Solvent extraction (i.e. liquid-liquid extraction) is a method used to separate a compound based on differential solubility in two immiscible liquids (e.g., water and an organic solvent), and it is widely used to separate CDs from the reaction mixtures. Organic solvents extract the amphiphilic CDs from unreacted reagents present in aqueous solution [88–90]. More indeed, a "gradient extraction" method using four organic solvents with different grades of polarity (hexane, carbon tetrachloride, carbon tetrachloride and dichloromethane, and dichloromethane) was applied by Han et al. to separate CDs synthesized from cow milk, according to the surface polarity of the CDs species [91]. As a consequence, the fractions obtained showed different surface polarities and surface polarity-dependent photoluminescence. However, it was concluded that this approach could not separate for an exact analysis of the hidden properties of each CDs species [77].

Nevertheless, the purification of CDs is not as simple as expected due to their high hydrophilicity and reduced size, which hinders their separation using filtration or centrifugation. On the one hand, dialysis presents some drawbacks: relatively high cost and time-consuming, difficulties to be scaled up and to separate potentially present large nanostructured by-products such as polymers [92] which, in turn, need to be removed by using membrane filters with a relatively high pore diameter (0.2 µm) [93]. Chromatography has been considered and applied for CDs purification, though it implies elevate costs. Alternatively, solid-phase extraction using alumina as solid phase and organic solvents for the extraction was applied to separate and purify violet, blue, green and yellow emitted CDs, thus reducing costs and time expenses [92]. The reaction mixture (aqueous or ethanolic solution of precursors heated at 150ºC) was transferred to a handmade solid-phase extraction column of neutral aluminium oxide in a glass tube, 1 cm diameter, 2 cm height and washed extensively with the organic solvent. CDs were extracted from alumina again using water or ethanol. This method used a simple thin layer chromatography (TLC) test to select the optimal washing solvent, which also permits the procedure to be adapted to other kinds of CDs [92]. Likewise, a method for the preconcentration and quantification of GQDs in aqueous samples using a strong anion exchange sorbent by solid-phase extraction has great potential to be applied to the determination of graphene quantum dots at trace levels in aqueous samples. The silica sorbent

was composed by a quaternary amine (functionalized with a chloride counter ion), packed in a polypropylene column. GQDs interacted with the charged sorbent through ionic interactions and thus can be preconcentrated for their subsequent elution [94].

Role of C-dots in separation techniques for bio(analytical) applications

Several reviews have been previously published about the numerous current applications of CDs [5,22,95,96], especially as of their particular optical properties, high water solubility, the surface defects that allow them to interact with other molecules, their potential surface passivation [97] and, obviously, their easy and low-cost synthesis. Additionally, they show low cytotoxicity, biodegradability and biocompatibility. CDs can penetrate easily through cell membrane, what makes them valuable to be applied to biology and bio-imaging [1,9,95,98], as potential therapeutic or antimicrobial agents [99,100], for gene or drug delivery applications, quantitation of biomolecules and sensing (i.e. photoluminescence, electrochemical, electrochemiluminescence sensing) only in bioanalytical fields, as well as in other sectors (e.g. environmental, energy, or science of materials) [5,101–103].

These aforementioned features of CDs contribute to the tremendous analytical and bioanalytical potential of these nanomaterials. Amongst all the vast potential applications that currently appear, this section will focus on different analytical techniques applied with bioanalytical purposes in which CDs are playing a significant role. For determining qualitative or quantitatively any analyte (i.e. with clinical, forensic, biomedical, or pharmaceutical purposes), it is essential to possess an effective separation technique, usually coupled with robustdetection systems, and it is also frequently required an adequate pre-treatment procedure, as many of the analytes are contained in low amounts within a complex matrix [104]. In this sense, there are several capillary-based separation techniques of particular importance, such as capillary chromatography, capillary electrochromatography (CEC), and CE, as they have shown to provide high separation power along with reduced turnaround time and low consumption of samples, especially in the microfluidic format [104].

Electrophoresis

The limitations associated to the use of CE, such as high limits of detection when using not too sensitive detection systems or low separation performance for certain applications, can be overcome by using specific nanomaterials like CDs. However, up to now, very few analytical methods based on separation techniques using CDs have been reported (Table 1). In particular, Sun et al. reported the use of GQDs of uniform particle size of 2.3 nm in the buffer solution as an additive for improving the efficiency in the separation of compounds of cinnamic acid and derivatives, which have a tremendous potential clinical application as antioxidants, antimicrobials and to prevent cancer. These analytes could interact with GQDs through π-π electrostatic stacking or hydrogen bonding interactions, thus modifying the electrophoretic velocity (increasing the mass-to-charge ratio) of the analytes to slow down their migration to improve selectivity. Several factors were analysed and optimized (concentration of background electrolyte, pH, separation voltage, injection time, the concentration of GQDs) to get an optimal separation of the analytes and detection by CE-UV within 18 minutes [105].

On the other hand, the improvement of sensitivity is essential in CE, more particularly to be comparable to less affordable equipment like chromatographic techniques combined with detection by mass spectrometry. Consequently, the application of CDs to enhance this sensitivity of CE should be considered, especially in those

laboratories in which only the more economic CE equipment is present. In this sense, Lahouidak et al. developed and validated a simple CE-FD method based on GQDs acting as emission enhancer of seven fluoroquinolones (lomefloxacin, norfloxacin, ofloxacin, ciprofloxacin, difloxacin, oxolinic acid and flumequine), antibiotics whose monitoring is essential. The synthesis of GQDs with relatively uniform diameter size and excellent photoluminescence properties was carried out by typical top-down methodology based on the size reduction of graphene followed by hydrothermal etching route using concentrated nitric acid and sulfuric acid as oxidants, producing a non-toxic material. The method was optimized concerning the type and concentration of BGE, pH and voltage, thus obtaining limits of detection, for ofloxacin, close to 11 ng mL^{-1}, similar to previous HPLC methods analysing ofloxacin, which confirmed the utility o GQDs to enhance the sensitivity of ofloxacin determination in milk samples [106].

Likewise, to demonstrate the applicability of CDs as separation adjuvants, mixtures of holo-(metallated) and apo- (demetallated) conformations of transferrin were analysed [107]. Briefly, only after the addition of CDs to the separation buffer, the selected forms of transferrin were separated by CE-FD. The production of CDs was carried out by pyrolysis of citric acid and other organic precursors. Fluorescence studies were performed to assess the interaction between CDs and apo- and holo-transferrin. A process of optimization was performed regarding sample preparation, buffer composition, ionic strength, pH, and temperature. The addition of CDs was tested either in the sample preparation and the separation buffer, being their presence in both steps simultaneously the configuration in which best performance was found. Nevertheless, different rate of association of the various forms of transferrin with CDs was observed by different extents of fluorescence quenching and remarkable changes in electrophoretic mobility for each species.

Chromatography

Reversed-phase liquid chromatography mode can separate most of the compounds, except many of the polar and hydrophilic compounds, as these compounds are weakly retained in the stationary phase. Hydrophilic interaction chromatography (HILIC) represents a real alternative, as like in normal-phase liquid chromatography, a polar stationary phase and a hydrophilic mobile phase are used, so hydrophilic compounds can be separated. In this sense, traditional materials (e.g. amino or bare silica gel) have been usually applied, although these are not always suitable to separate specific compounds.

CDs are small in size, water-soluble, and easy to be functionalized so that CDs can be regarded as potentially useful elements as chromatographic separation materials in liquid chromatography (Table 1). Yuan et al. recently prepared CDs from glucose and potassium dihydrogen phosphate by sonication after adding water, transferred to an autoclave and then the oxygen removed with nitrogen. The reaction was heated at 200°C for 12 h, and then centrifuged and filtrated. After 10 h of dialysis for 10 h, the dialysis solution was freeze-dried, and the product re-dispersed in ethanol to eliminate the potassium dihydrogen phosphate and the supernatant was dried by rotary evaporation. Finally, the Glc-CDs was obtained through freeze-drying, and the Sil-Glc-CDs and Sil-Glc were packed into the stainless steel column through the slurry method [108]. Nucleosides and nucleobases were analysed, and the acetonitrile content, buffer concentration, mobile phase pH and column temperature were optimized to improve the HILIC chromatographic separation. It was applied to separate and quantify fructose and glucose from goji berry aqueous solution. This stationary phase separated multiple polar

compounds with higher selectivity than other commercial and non-CDs modified columns. The separation performance was observed to be influenced by the partitioning and adsorption mechanisms.

Previously, Zhang et al. synthesized N-doped CDs prepared via a solid-phase synthesis approach to decorate silica particles which were packed into chromatographic columns, to study their performance as adsorbent material for liquid chromatography. They were confirmed to be selective for polar compounds in HILIC chromatography [109]. Similarly, glucose-based-nitrogen-doped CDs, prepared by pyrolyzing glucose and aspartic acid, and then grafted on silica surface via isocyanatopropyl as linker using deep eutectic solvents (DES) as reaction medium, were packed in stainless columns for HILIC separation. The effects of acetonitrile content, buffer salt concentration, buffered pH and column temperature on the retention of nucleosides and bases were analysed to ascertain the retention mechanism of this modified stationary phase. The separation of amino acids, ginsenosides, saccharides and antibiotics all showed good performance with this phase, thus confirming its remarkable hydrophilic properties. More indeed, separation selectivity using this stationary phase was improved for roxithromycin determination in the capsule when compared with non-doped Sil-Glc-CDs column, mainly due to the effect between the functional groups of these so-synthesized CDs, thus increasing the interaction sites with analytes [110]. Likewise, p-phenylenediamine (PPD) was prepared into carbon dots (PPDCDs) by a solvothermal method. These CDs were reacted with a silanization reagent and then grafted onto bare silica gel by a one-pot method in DES. This new so-modified silica gel (Sil-PPDCDs) was used as stationary phase, which was successfully applied to separate again nucleosides and bases, amino acids, saccharides from *Lycium barbarum*, which possess potential medicinal function, and ginsenosides [111]. Also, to enhance the performance of hydrophilic interaction liquid chromatography by increasing the density of surface hydrophilic functional groups, Cai et al. proposed and studied the application of polyethyleneimine (PEI) as the hydrophilic precursor to produce polyamine-functionalized carbon dots (PEICDs), since PEI contain much more amine groups and PEI-functionalized carbon dots (PEICDs) mix-grafted silica packing material as the stationary phase. They demonstrated a synergy within the co-immobilized stationary phase, which showed better column efficiency compared to that of Sil-PEI, as well as enhancement of the retention ability and selectivity at the analysis of 11 nucleosides and nucleobases and 9 ginsenosides [112].

Nevertheless, CDs are not only applied to HILIC chromatography, but also their potential role in normal and reversed-phase chromatography is explored. Wu et al. developed a new column able to perform good chromatographic separations, not only of the polar compounds thymidine, uridine, adenosine, cytidine and guanosine but also of anilines, phenols and polycyclic aromatic hydrocarbons in normal and reversed-phase mode [113]. A combination of different retention mechanisms was observed for this stationary phase: adsorption and electrostatic interaction in hydrophilic chromatography mode, as well as π-π stacking and π-π electron-donor-acceptor interactions. Briefly, GQDs were prepared from graphene oxide aqueous dispersion using a hydrothermal synthesis method, which specifically required H_2O_2 and ammonia, as an etching agent and assistant, respectively. The different aromatic compounds analysed were correctly separated using the new stationary phase formed by GQDs/SiO_2 in both normal and reversed-phase modes.

Additionally, the amount of methanol required is relatively low, thus representing a significant advantage in economic and environmental terms. On the other side, six alkaloids, five nucleosides and four nucleobases were successfully determined and discriminated in HILIC mode. In this mode, it was found that this column

established a complex retention mechanism which includes partitioning, adsorption and electrostatic interactions. Again, and taking advantage of the numerous hydroxy and carboxyl groups of GQDs, which give them the capacity of establishing hydrophobic, hydrophilic, π-π stacking and hydrogen bonding, Wu et al. developed, by covalent bonding method, another novel stationary phase composed by octadecyl modified GQDs-bonded silica. was also applied in reversed-phase and hydrophilic interaction liquid chromatography analysis. This new column showed better separation of certain compounds when it was compared with commercial C_{18} column [114].

Alternative substances such as ionic liquids (ILs) have also been supported on silica gel to create new stationary phases with the ability to create multiple interactions used for HILIC chromatography. More indeed, and to exploit the adjustable surface functionalization of CDs, the integration with ILs will create new possibilities not observed up to now. That is the case for the imidazolium ionic liquid-derived CDs (ImCDs), which were recently prepared and coated onto porous silica microspheres. This new phase improved the selectivity of the separation for UV detection of different probe analytes (sulphonamides, amino acids, sugars, ginsenosides, nucleosides and nucleobases), mainly due to the existence of abundant functional groups such as imidazolium, carboxyl acid, and hydroxyl groups [115]. It is noteworthy that this new column does avoid severe peak tailing and low column efficiency previously found with other carbon nanomaterial functionalized silica stationary phases, thus representing impressive alternative materials to be applied in many applications.

The separation of enantiomers of chiral compounds represents a significant challenge for HPLC techniques, primarily because of the interest and the critical role that chiral molecules and enantiomers play in the biological, pharmaceutical and biomedical field. However, very few advances have been published regarding the application of CDs to enhance the chiral separations by HPLC using chiral selectors such as cyclodextrins. Interestingly, it has recently been developed GQDs functionalized β-cyclodextrins (CD) and cellulose silica composites as chiral stationary phases to analyse the potential effect of GQDs on chiral separation. It was tested for ten chiral compounds and compared with β-CD and cellulose chiral stationary phases. 3,5-dimethylphenyl isocyanate is very frequently utilized as derivatization reagent of β-CD and cellulose and, therefore, it was also observed the difference between GQDs and this derivatizing agent regarding chiral separation performance [116]. GQDs-functionalized β-CD and cellulose columns showed stronger retention ability than β-CD and cellulose columns. These authors found that GQDs enhanced the effect for all the compounds analysed. At the same time, 3,5-dimethylphenyl isocyanate only showed an enhancement effect for four chiral compounds, which was explained as of the introduction of extra-interactions by GQDs (i.e. π-π and hydrogen-bond) as well as of the spatial structure of this nanomaterial, that influences the surrounding environment of chiral selectors.

The properties mentioned above of CDs make them also potentially useful for their application in gas chromatography, even though almost no previous work has utilized them. Zhang et al. explored the use of GDs as the stationary phase for gas chromatographic capillary column separation of alkanes and aromatic isomers with 3-aminopropyl-diethoxy methyl silane (3-AMDS) as coupling reagent [117]. The supramolecular interaction (mainly the CH-π interaction), the π-π conjugated network and surface groups offered abundant binding sites for the analytes. Additionally, a reduction of the particle size of the stationary phase improves mass transfer in gas chromatography. Even though this group did not use this approach for bioanalytical purposes, they

successfully immobilized GQDs onto the capillary wall using 3-AMDS as a coupling reagent to evaluate the chromatographic performance to separate alkanes, ethylbenzene, styrene, propyl benzene, dichlorobenzene and xylene isomers. High resolution, good selectivity, and reproducibility, within 9 minutes at low temperature without the process of temperature-programming were obtained.

Role of C-dots in extraction techniques

Sample pre-treatment is a necessary and determinative step in the analysis, to isolate the analytes from the rest of compounds within a sample to favour their detection, to improve the sensitivity and/or the selectivity of the methodologies utilized. In this sense, solid-phase extraction (SPE), solid-phase microextraction (SPME), dispersive solid-phase microextraction (DSPME), and solvent phase, namely liquid-liquid extraction (LLE), have been extensively used in last decades. The role of CDs in these techniques and the application of membranes are here discussed, as CDs are widely used in sample preparation owing to the surface of the CDs contains a significant number of functional groups, which improve the hydrophilicity and dispersibility of the material (Table 1) [108].

A new nano-sorbent for solid-phase extraction was developed by immobilizing CDs on the microcarrier cytopore, creating the so-called C-dots@cytopore, whose performance was tested for the adsorption of heavy metals such as cadmium, which is hardly metabolized and highly toxic, even at very low concentration levels. CDs with large surface area provide more active sites to that provided by the bulky adsorbent, facilitating fast adsorption and desorption dynamics, and the effects of the acidity of the sample solution, the eluent's volume and concentration, as well as interfering effects, were studied. The composites showed higher adsorption property, reaching the adsorption equilibrium in just 1 min, thus allowing a rapid separation, enrichment and detection of cadmium in environmental samples at trace levels [118]. Moreover, aflatoxin B1, recognized as a group I carcinogen by the International Agency for Research on Cancer (IARC) is one of the world's three most potent carcinogens, requires of accurate and precise detection, as different countries and organizations have set legal limits. With this aim, 5,7-dimethoxycoumarin was used as a dummy template for aflatoxin B1, methacrylic acid as functional monomer, ethylene glycol dimethacrylate as cross-linker and azobisisobutyronitrile (AIBN) as initiator, to dope MIP with CDs, to prepare a molecularly imprinted monolithic column by in-situ polymerization. Thus, high specificity, as well as the large specific surface area, were obtained to separate and detect this toxin. The analyte's extraction performance of the CDs-DMIP column was evaluated concerning recovery, precision, column capacity, and enrichment effect [119]. More interestingly, new CDs have also been applied as SPE sorbent, in this case, to analyse five different parabens and organophosphorus pesticides by LC-MS/MS. CDs were created using a green synthesis protocol, and the method was optimized and validated, obtaining good analytical performance and optimal recoveries (63–123%) for all analytes from effluent water samples, thus showing CDs as an effective sorbent for these different analytes without much modification [120].

SPE techniques, however, have shown in some cases, low efficiency, a not easy to separate adsorbent, and time-consuming approaches. As a consequence, new and more straightforward pre-treatment techniques (e.g. magnetic solid-phase extraction-MSPE, or dispersive solid-phase extraction-DSPE) have been developed to overcome these limitations [121]. MSPE employing magnetic nanoparticles (NPs) as adsorbents, possess large surface area, short diffusion pathway and ease of separation as the sorbent does not need to be packed into the cartridge and the phase separation can be easily realized by applying an external magnetic field, thus

reducing the time of operation without losing extraction efficiency. Nevertheless, these NPs need to be functionalized to avoid their aggregation. Hence, CDs represent a right choice as they are mainly formed by sp^2 and sp^3 carbon atoms that allow them to provide adsorbents with strong affinity by π–π stacking interaction and hydrophobic interaction towards analyte to be analysed. For instance, polycyclic aromatic hydrocarbons (PAHs) are highly toxic, as of their teratogenicity, mutagenicity and carcinogenicity. These compounds were analysed to evaluate the potential of CD@PGMA@Fe_3O_4 NPs for MSPE assisted by microwave, and using poly(glycidyl methacrylate) (PGMA) as the grafted branch to enhance and control the loading amount of CDs, thus allowing to control extraction performance. This extraction approach together with HPLC–UV/Vis detection, represents a novel analytical method for PAHs determination [122].

Additionally, Fe_3O_4/N-CDs was synthesized to determine Pb^{2+}, a highly toxic element, from different biological and environmental samples using the MSPE method before its detection by atomic absorption spectrometry [123]. Besides, Fe_3O_4/hydroxyapatite/GQDs nanocomposite was synthesized to pre-concentrate copper residues, a micronutrient which, also, could be a highly toxic and non-degradable pollutant, from food samples to be subsequently determined by inductively coupled plasma-atomic emission spectrometry [124]. This extraction, microwave-assisted, was optimized with regard to pH, time of adsorption and desorption, the solvents used for elution, and the amount of nanomaterial required, and remarkable good figures of merit related to the linear range (0.05–1500 ng mL^{-1}), limits of detection and quantification below 0.6 and 2 ng mL^{-1} respectively, as well as of precision and recovery (83.5-104.8%) were obtained, thus representing an acceptable option for determining this heavy metal in food samples, even more considering its reusability just after a simple treatment with deionized water. Alternatively, to the previous approaches to extract and determine toxic heavy metals, a novel SPE adsorbent of hollow calcite single crystals prepared by an etching technique, via the precipitation of metal nitrates by the CO_2 diffusion method, in the presence of CDs, was created to increase the specific surface area to increase the adsorption ability of calcite for heavy metals adsorption. This adsorbent was applied to remove the excess of Cd(II) ions from wastewater. CDs were first prepared through the one-pot hydrothermal route of the propyl aldehyde and sodium hydroxide via an aldol condensation reaction [125]. The maximum adsorption capacity was obtained at pH 5 after 90 minutes, a process consisting of two consecutive steps: initial chemisorption followed by slow removal indicating surface precipitation.

About pharmaceutical products determination in biological matrices, which are very frequently analysed using separation techniques, CDs have been scarcely applied to their prior extraction from complex matrices. In this sense, a magnetic CD/graphene oxide hybrid material, obtained by a green production method from pasteurized cow milk, was applied as an adsorbent to extract ibuprofen from human plasma to be then determined by HPLC-DAD [126]. Briefly, to avoid restacking and aggregation of the GO, CDs were dispersed into carbon layers of GO. Under optimized experimental conditions of sample solution pH, amount of adsorbent, extraction time, eluent type and a limit of detection of 8.0 ng mL^{-1} and recoveries ranging from 91% to 95% with relative standard deviation lower than 4% were obtained, thus providing a fast and straightforward approach to monitor this drug in human plasma samples. Additionally, the extraction performance of this material remained stable up to twelve cycles of MSPE. DSPE possesses many advantages such as the convenience of operation, reduced solvent consumption and time required, and dispense with special equipment in sample treatment. Developing an appropriate sorbent in DSPE procedure as it increases the active surface area with the analytes, thus achieving better extraction performance [127].

Microextraction reduces, not only the amount of solvent extraction required but also the time needed to extract the analytes. In this sense, Liu et al. designed and developed a CD-based SPME fibre for the selective screening, by fluorescence quenching state of the CDs after extraction, and sensitive detection of 2-nitroaniline in urine and environmental samples [128]. Urea and calcium nitrate were used as precursors in the formation of the N-doped CDs, contributing to positive (amino) and negative (carboxyl and hydroxyl groups) surface functional groups, respectively. The presence of these functional groups on the CDs and the negatively charged CDs surface may promote the hydrogen bonding interaction and electrostatic interaction with the amino group of nitroaniline, thus causing quenching of emission intensity.

More indeed, magnetic dispersive solid-phase microextraction (MDSPME) has been successfully applied to extract and determine, by GC-MS, organophosphorus pesticides, which can affect the nervous system in humans and other animals, in aqueous and food samples [129]. Specifically, in MDSPME, the magnetic sorbents are ultrasonically dispersed into the sample solution to create a cloudy suspension able to extract and enrich the target analytes. Once accomplished the extraction, the sorbent is separated from the aqueous sample by applying an external magnet, then the extracted analyte is desorbed and can be accordingly analysed. In this method, pH and volume of donor phase, stirring rate, extraction time, as well as the type, volume of solvents and time of desorption were analysed for optimizing the extraction procedure. Good analytical performance was observed, although it was calculated higher relative standard deviations compared with previous strategies using solid-phase extraction coupled to GC methods.

On the other hand, dispersive liquid-liquid microextraction (DLLME) represents an adequate alternative to extract compounds for their analysis, although it has been scarcely applied including CDs, even though their potential advantages and applications. In this sense, only fenitrothion (FNT), used in agriculture as a pesticide and sometimes used to support public health against diseases such as malaria, has been extracted by DLLME using CDs. The residues of FNT should be monitored, as they may contaminate canal sources, thus polluting aquatic organisms and, ultimately, putting human health into risk through food chains [130]. In this only application, up to now, of CDs to DLLME, unmodified CDs were extracted into the organic phase, exhibiting intense absorption signal at 260 nm. On the other hand, when FNT is present, the absorbance signal of the enriched phase increased as of the FNT-CDs interaction at the colloidal interface of the nanomaterial. This increase in the absorbance of the CDs in the organic phase was utilized to quantify FNT.

For membranes, there is a recent interest in their potential to separate small organic molecules (e.g. pharmaceuticals). More specifically, different membrane designs included CDs in their designs: reverse osmosis (RO), ultrafiltration (UF), nanofiltration (NF), forward osmosis (FO), pressure retarded osmosis (PRO), membrane distillation (MD), and organic solvent nanofiltration (OSN). In particular, nanofiltration represents a membrane separation technology between the UF and RO techniques because of its pore size, flux, and operating pressure [131]. Achieving selective separation as well as a good permeability is the most critical requirement for the future of membrane separations. In this sense, nanoparticles have been introduced into membranes via a mixed matrix approach to improve selectivity and surface properties. According to the application of the membrane, they can be selectively engineered only the dense-selective layer, the substrate layer or the entire membrane by CDs [132]. CDs, due to their economic and uncomplicated production, as well as their physicochemical properties, have recently attracted extensive attention in the field of membrane technologies for their applications in separation processes. For instance, a stable membrane that incorporated

graphene oxide quantum dots (GQDs) into a cellulose membrane using an ionic liquid (1-ethyl-3-methylimidazolium acetate) to remove small molecules (> 300 Da). This membrane possesses numerous surface hydroxyl and carboxyl groups, a negative surface charge of GQDs to avoid aggregation. Furthermore, GQDs are smaller than 5nm, thus resulting in the higher edge area and high functionality per the mass of a particle. GQDs are located on the membrane surface to give the membrane negative surface charge, and improved hydrophilicity and they are bound to the cellulose thanks to hydrogen bond networks, giving to the resulting membrane stability under convective flow and shear stress. GQDs showed no leaching after convective flow through the membrane. Impact of GQD on membrane permeability and rejection was studied through convective flow experiments, and through longer-term permeability studies [133]. These GQDs-cellulose membranes performed their function in a mode between ultrafiltration and nanofiltration, as they can separate dyes between 300 and 10^3 Da.

Presence of hydrophilic functional groups besides their high surface area, chemical stability, nontoxicity, and good dispersion in organic solvents makes carbon-based nanomaterials highly effective nanofiller, so nitrogen-doped CD-blended PES membrane polymer was fabricated through the phase inversion method and observed to possess high potential as hydrophilic and antibacterial nanofiller [131]. Previously, it was first reported and its antimicrobial and anti-biofouling capacity tested of a GQDs covalent-functionalized PVDF membrane. The presence of the GOQD coating layer effectively inhibited the growth of the bacterial cells and prevented the formation of the biofilm on the membrane surface [134]. Nevertheless, more extensive review related to membrane technologies and CDs has been published elsewhere [132], in which it is reinforced the idea of further research required on the process of formation of these membranes, anti-fouling mechanisms, biofilm growth and toxicity.

Table 1. Bioanalytical applications of C-dots playing a key role in different separation techniques.

Analytical Technique	Role of NM	C-dots synthesis approach	Analyte(s)	Application	Reference
Capillary electrophoresis					
	Additive	Flake graphite powder by modified chemical oxidization and purified by macroporous resin column	Cinnamic acid, 3,4-dimethoxycinnamic acid, 4-methoxycinnamic acid, Isoferulic acid, Sinapic acid, Ferulic acid, trans-4-hydroxycinnamic acid	Checking of utility of GQD at CE performance (standard sols.)	[105]
	Emission enhancer	Size reduction of graphene followed by hydrothermal etching route using concentrated nitric acid and sulfuric acid as oxidants	Lomefloxacin, Norfloxacin, Ofloxacin, Ciprofloxacin, Difloxacin, Oxolinic acid and Flumequine	Analysis of ofloxacin in milk samples	[106]
	Sample extraction / separation buffer additive	Oven pyrolysis of citric acid	holo-(metallated) transferrin, apo-(demetallated) transferrin	Improvement of metalloprotein mixtures analysis	[107]
Liquid chromatography					
Reversed or normal phase					
	GQDs as a component of a novel stationary phase.	GO aqueous dispersion was synthesized from natural graphite powder using a hydrothermal synthesis method with H_2O_2 as etching agent and ammonia. Dilution with deionized water and transferred to autoclave at 180ºC for 8 h. Then, filtration through nylon membrane, heating of supernatant to remove unreacted ammonia and H_2O_2. Finally, freeze-drying.	Anilines, phenols and polycyclic aromatic hydrocarbons	Investigation of the chromatographic performance and retention mechanism of GQDs/SiO$_2$ column in reversed and normal phase modes	[113]
	GQDs as a component of a novel stationary phase.	GO aqueous dispersion was synthesized from natural graphite powder using a hydrothermal synthesis method with H_2O_2 as etching agent and ammonia.	Anilines, phenols, alkylbenzenes and polycyclic aromatic hydrocarbons	Investigation of the chromatographic performance and retention mechanism of C_{18}/GQDs/SiO$_2$ column in reversed and normal phase modes	[114]
HILIC mode					
	CDs-modified silica stationary phase	Glucose and potassium dihydrogen phosphate mixing and sonication after water addition, transference to autoclave and oxygen removal with N_2. Reaction heating at 200°C for 12 h, centrifugation and filtration. After dialysis for 10 h, the dialysate was freeze-dried, and the product re-dispersed in ethanol. The potassium dihydrogen phosphate	Nucleosides, nucleobases, ginsenosides, amino acids, antibiotics, and saccharides	Separation of polar compounds and comparison with commercial and non-CDs modified phases.	[108]

Analytical Technique	Role of NM	C-dots synthesis approach	Analyte(s)	Application	Reference
		is precipitated and the supernatant dried by rotary evaporation, then Glc-CDs was obtained through freeze-drying			
	Adsorbent material for chromatography	Solid-phase synthesis approach: Mixture and grounding of Tryptophan and aconitic acid. Transference of the mixture into autoclave and heated at 220°C for 6 h, then purified and freeze-dried.	Nucleosides and nucleobases in *Cordyceps* sample	Evaluation of the chromatographic performance of Nitrogen doped CDs-modified silica as HPLC stationary phases	[109]
	Glc-N-CDs-modified silica stationary phase	Grafting on silica surface from glucose and aspartic acid, previously pyrolyzed at high temperature, via isocyanato-propyl as linker using deep eutectic solvents as reaction medium.	Amino acids, ginsenosides, saccharides and antibiotics	Evaluation of the chromatographic performance of GLC-N-doped CDs-modified silica as HPLC stationary phase for polar and hydrophilic analytes determination	[110]
	p-phenylenediamine-derived CDs were decorated on silica spheres as new stationary phase	Solvothermal method with some modifications: *p*-phenylenediamine was dissolved in ethanol, the solution was transferred into autoclave and heated at 160ºC for 2 h. Concentration of the products by rotary evaporation and dried under vacuum at 60 °C to obtain PPDCDs.	Amino acids, sugars, ginsenosides, saccharides in *Lycium barbarum*	Evaluation of the chromatographic performance of Silica-PPDCDs stationary phase for polar compounds determination	[111]
	PEI and PEICDs, mix-grafted silica packing material as stationary phase	PEICDs were prepared by one-step pyrolysis of citric acid in PEI aqueous solution. The resulted mixture was dialyzed using cellulose ester membrane bag to remove unreacted PEI. The purified CDs suspension was vacuum distilled and lyophilized to get dried PEICD.	Nucleosides, nucleobases and ginsenosides	Analysis of chromatographic performance of a stationary phase in which PEICDs were stabilized on silica individually, and simultaneously	[112]
	Component of a novel stationary phase.	GO aqueous dispersion was synthesized from natural graphite powder using a hydrothermal synthesis method with H_2O_2 as etching agent and ammonia. Mixture dilution with deionized water and transferred to autoclave at 180ºC for 8 h. Then, filtration through a nylon membrane, heating of the supernatant to remove the unreacted ammonia and H_2O_2. Finally, freeze-drying.	Alkaloids, nucleosides and nucleobases	Investigation of the hydrophilic performance of this stationary phase in HILIC mode.	[113]
	GQDs as a component of a novel stationary phase.	GO aqueous dispersion was synthesized from natural graphite powder using a hydrothermal synthesis method with H_2O_2 as etching agent and ammonia.	Alkaloids, nucleosides and nucleobases	Investigation of the hydrophilic performance of this stationary phase in HILIC mode.	[114]

Analytical Technique	Role of NM	C-dots synthesis approach	Analyte(s)	Application	Reference
Chiral separation	CDs-Imidazolium-modified silica stationary phase	[AEMIm][Br] and citric acid monohydrate (molar ratio 2:1) were mixed and dispersed in water. Then the mixture was transferred into autoclave and heated at 200ºC for 4 h.	Nucleosides and nucleobases, Sulphonamides, Amino acids, saccharides, and ginsenosides.	Analysis of the chromatographic behavior of the proposed stationary phase.	[115]
	GQDs functionalized β-CD and cellulose silica composites were first applied in HPLC enantioseparation	GO aqueous dispersion was synthesized from natural graphite powder using a hydrothermal synthesis method with H_2O_2 as etching agent and ammonia. The mixture was diluted with deionized water and transferred to autoclave at 180ºC for 8 h. After that, Filtration through a nylon membrane, heating of the supernatant to remove the unreacted ammonia and H_2O_2. Finally, freeze-drying.	Chiral racemates benzoin, benzoin methyl ether, benzoin ethyl ether, 6,6'-dibromo-1,1'-bi-2-naphthol, trans-stilbene oxide, flavanone, 6-hydroxyflavanone, naphthyl ethanol, diclofop, metalaxyl	To investigate the effect of GQDs on chiral separation	[116]
Gas chromatography	Stationary phase – capillary coating	Synthesis from natural graphite powder: GO dispersed in deionized water with hydrogen peroxide, re-oxidation by ozone, transferred to autoclave (50 mL) and heated 200ºC. Filtration of the suspension through a 0.22 μm microporous membrane.	Alkanes, ethylbenzene, styrene, propyl benzene, dichlorobenzene and xylene isomers	Analysis of GQDs as the stationary phase for gas chromatographic capillary column separation of alkanes and aromatic isomers with 3-aminopropyldiethoxymethyl silane as coupling reagent	[117]
Extraction techniques					
Solid phase extraction	Element of composite used as sorbent	One-pot hydrothermal route. EDTA-Na$_2$ was dissolved in DI water with ultrasonic treatment. The solution was transferred into autoclave and heated at 200°C for 12 h. The reaction was cooled down to ambient temperature, the solution was filtered through a 0.22 μm membrane to remove impurity.	Cadmium	Optimization of the SPE Extraction and determination of Cadmium in environmental samples	[118]
	Element of new monolithic column (CD-MIPs)	N-(β-aminoethyl)-γ-aminopropyl methyl dimethoxy silane was added to a three-neck flask purged with N$_2$ for 5 min, heated to 240ºC, and rapidly added 0.5 g anhydrous citric acid under vigorous stirring, for 5 min, and then cooled to room temperature. CDs were 3-times precipitated with petroleum ether, and the precipitate redissolved in methanol.	Aflatoxin B1	Study of analytical performance of CD-MIP composite for aflatoxin B1 determination.	[119]
	CDs as SPE sorbent	Oat grains are weighed, crushed, and pyrolyzed at 400°C for 2 h in a muffle furnace. The product was cooled at ambient temperature before being mechanically	Methylparaben, ethylparaben, propylparaben, azinphos-methyl and parathion-methyl	Application of pristine CDs as SPE sorbent for developing and validating a method to preconcentrate and determine pesticides and parabens in wastewater samples	[120]

Analytical Technique	Role of NM	C-dots synthesis approach	Analyte(s)	Application	Reference
	Component of composite as new adsorbent for MSPE	crushed to fine powder, which is dispersed in ultrapure water and centrifuged at 7800 rpm several times. The aqueous suspension was filtered and the CDs residue dried for 24 h at 80 °C. Poly(ethylene glycol) and saccharide were added to distilled water to form a solution that was heated in a 500 W microwave oven for 2–10 min. The solution changed from colourless to dark Brown, which implied the formation of CDs.	Polycyclic aromatic hydrocarbons	Evaluation of MSPE extraction performance of the proposed composite.	[122]
	MSPE Sorbent composed by Fe_3O_4 and N-doped CDs	Citric acid and dicyandiamide were dissolved into 5 mL water, and stirred. The solution was transferred into a stainless autoclave and heated to 160°C for 4 h. The final product was collected adding ethanol and centrifuged at 5000 rpm for 5 min.	Lead	Optimization of extraction performance of Fe_3O_4/N-CDs composite for determining lead in aqueous and vegetable samples	[123]
	MSPE sorbent composed by Fe_3O_4, hydroxyapatite and GQDs	Citric acid was heated at 200°C on a paraffin oil bath for 5 min. The resulting liquid was added dropwise to 20 mL NaOH solution (0.25 M) while vigorously stirring at room temperature. After that, the obtained GQDs aqueous solution was stored at 4°C in amber bottle	Copper	Preparation, characterization, and application of Fe_3O_4/HAP/GQDs magnetic nanocomposite for an ultrasound-assisted MSPE for the pre-concentration and determination of Cu in food ingredients	[124]
	Component of MSPE adsorbent hybrid composite Fe_3O_4@CD@GO	20 mL of deionized pure water was added to 25 mL of pasteurized cow's milk and the mixture was transferred to hydrothermal synthesis unit, in which a Teflon vessel was placed. The mixture was allowed to react at 180ºC for 12 h and CDs obtained were separated from the liquid phase by centrifugation (4000 rpm for 10 min.), washed twice with purified water, once with ethanol and dried 75ºC.	Ibuprofen	Optimization and validation of analytical methodology for ibuprofen extraction and determination in human plasma	[126]
	Element of a novel SPE adsorbent, formed by hollow calcite single crystals in the presence of CDs	Sodium hydroxide was slowly added into10 mL propyl aldehyde solution under stirring at 20°C for 6 h. The tawny colloidal solution obtained was laid in a sealed container for 5 days. The tawny solid was rinsed with 15 mL of 0.5 M nitric acid to form yellow turbid liquid, which was centrifuged for 15 min at 4000 rpm and dried at 60 °C for 6 h.	Cadmium	Analysis of the extraction capacity of the proposed adsorbent of cadmium from wastewater samples.	[125]

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Analytical Technique	Role of NM	C-dots synthesis approach	Analyte(s)	Application	Reference
Solid phase microextraction					
	Part of a new SPME sorbent	DMF was added to graphene oxide (0.5 g) and ultrasonically treated for 2 h. The mixture was transferred into autoclave and heated at 250ºC for 8 h. The resulting mixture was filtered through a nylon membrane (0.2 μm) and heated to vaporize the excesses of DMF.	Sevin, fenitrothion, malathion, parathion, and diazinon	To develop a magnetic dispersive solid-phase microextraction for preconcentration and GC-MS determination of five organophosphorus pesticides in water samples	[129]
	Component of a fluorescent SPME fibre	N-doped CDs were prepared through a one-pot microwave heterogeneous reaction between calcium citrate and urea. Urea first decomposed and produced large amount of ammonia due to fast and efficient heating under microwave, which accelerated the decomposition of calcium citrate. The thermal carbonization of the precursors and the nucleation occurred in succession.	2-nitroaniline	Use of the fluorescent SPME platform for screening and quantification of 2-nitroaniline in environmental water samples and biofluids	[128]
Liquid-liquid extraction					
	Incorporation of CDs into extraction solvent	3 mL of NaOH solution (5 M) was added drop wise in 50 mL of filtered sugar cane juice under stirring for 15 min. Sonication for 30 min and centrifugation for 15min at 13,000 rpm. Upper clear solution (CDs) was separated.	Fenitrothion	Application of CDs in DLLME for determination of Fenitrothion in environmental water samples	[130]
Dialysis					
	Additive of NCDs to nanofiltration membrane	From ammonium citrate dibasic dissolved in double distilled water was moved into a Teflon-lined stainless still reactor and heated for 18 h 150 °C. The resulted solution was cooled down to room temperature, centrifuged and filtered using 0.22 μm membrane to obtain the pure N-CDs.	Salts and dyes	Analysis of the effects of adding NCD nanoparticles to PES membrane in terms of permeability, antifouling and antibacterial properties, and rejection performance.	[131]
	Modifier of cellulose membrane	GQDs were prepared from acetylene carbon black. Concentrated H_2SO_4 and HNO_3 solutions were added (2:1 vol ratio), under temperature monitoring. After attaching a reflux condenser, the round bottom flask was heated at 105 °C for 5 h. The GQDs solution was cooled down to room temperature, and deionized water was added. The solution was placed in ice bath and neutralized with KOH. Salts precipitated removed by vacuum filtration and remaining salts in the filtrate containing GQDs were removed by dialysis for	Dyes	Analysis of the performance, stability, and consistence of the composite membrane	[133]

Analytical Technique	Role of NM	C-dots synthesis approach	Analyte(s)	Application	Reference
		a week. Solid GQDs were obtained by drying the solution at low humid environment at 50 °C under vacuum.			
	Modifier of PVDF membrane	GQDs were synthesized with CX-72 carbon black via reflux in concentrated HNO$_3$ solution. CX-72. After cooling to below 30 °C, the mixture was diluted with water and filtered with a 10 kDa molecular weight cut-off membrane. Drying of percolate at 60°C.	*E. coli* and *S. aureus* cultures	Exploring the use as an antimicrobial agent and as an antibiofouling of membrane GQDs covalent-functionalized PVDF membrane.	[134]

CE: capillary, electrophoresis; DMF: dimethylformamide; Glc-CDs: glucose modified carbon dots; Glc-N-CDs: glucose-nitrogen doped-carbon dots; GO: graphene oxide; GQDs: graphene quantum dots; MIP: molecularly imprinted polymer; PEI: polyethyleneimine; PEICDs: polyethyleneimine modified carbon dots; PPDCDs: p-phenylenediamine-modified carbon dots; PVDF: polyvinylidene fluoride; SPE: solid phase extraction; [AEMIm][Br]: 1-aminoethyl-3-methylimidazolium bromide.

Pitfalls and future prospects

CDs, as an excellent fluorescent carbon nanomaterial, have been widely studied since it was first reported in 2004. In more than a decade, a large number of CDs have been synthesized through kinds of synthesis methods with significant differences in structure and performance characteristics [135]. While amorphous carbon-based nanoparticles without quantum confinement are called carbon nanodots, the crystalline structures and quantum effects are present in spherical CQDs or single-layer GQDs. However, classifying particles whose fluorescence is derived from both structure and molecular-like excited states remains a challenge, so their assignment is better preferred as graphitic or amorphous CDs, mainly because, in many works, there is a void of details referred to the synthesis steps, as well as because of incomplete process of characterization, inadequate or inconsistently reported purification [136]. Additionally, different phenomena like the aggregation of the carbonaceous materials and lack of reproducibility concerning the production of concrete particles' size, crystalline structures or surface functionalization, usually occur in both the top-down and bottom-up synthesis procedures, thus affecting the quality of these nanomaterials. In this sense, it exists a debate around the exact molecular weight of CDs, so further research involving original and new methods with improved resolution and sensitivity is warranted. Since their discovery, it has been confirmed that several green approaches are affordable and useful for CDs synthesis. However, the low yield usually observed implies that if large-scale synthesis of CDs is required shortly, more massive amounts of solvents and strong acids will be needed, thus compromising these eco-friendly synthesis approaches of CDs. Therefore, alternative carbon sources (e.g. biomass) must be considered [137], as well as the reproducibility is even more critical, even though most of the synthesis methods do not detail it. Thus, preparing high-quality nanomaterials based on rational design and quality control is currently required and a field to be intensively explored. In metrology and analytical chemistry, it is fundamental to be able to report on the traceability of the acquired results.

Method validation and quantification of measurement uncertainty represent two main factors for analytical quality control. To determine the accuracy of analytical methodologies, a certified reference material (CRM) of the same type as the samples and characterized to an acceptable standard about their identification, and quantitative determination of its components is required. Their use is of crucial importance for the reliability of results of chemical analysis, although standards or reference materials exist both as suspensions and as powders just for some nanomaterials, while additional reference materials are still under development. However, the use of these powdered nanoparticle standards does not include a standardized procedure for dispersion of the nanoparticles, which increases the uncertainty of the original metric indicated as several metrics such as size distribution depend on how this dispersion is carried out and under which conditions. In the absence of CRMs, there is also the possibility to use non-certified materials (test materials) to benchmark analytical procedures. Calibration standards used for quantification are generally traceable to a primary national or international standard. However, for nanoparticles, the validity of these standards has a shorter lifetime than most other standards and is more sensitive to operating conditions. Consequently, the synthesis and development of CDs standards and adequate reference certified materials become essential to perform an adequate qualitative and quantitative analysis of CDs, as well as for developing accurate and precise quality control studies [138].

Even though the properties of C-dots are currently much better understood, it still lacks a complete characterization of CDs mixtures, which are simply the addition of average properties of all individual CDs species present within. Consequently, further purification of the initial products synthesized utilizing advanced, precise and highly selective separation techniques must be accomplished to increase the current knowledge on the structural, photophysical, and chemical properties of this nanomaterial, and to minimize variability in their biological applications. Furthermore, quantitative analytical methods are required to determine CDs concentrations to analyse the effect and exposure assessments for these materials. Many methods still need optimization and development, as particular challenges for studies of CDs in biological samples occur: first, the detection limits for most methods are not sufficiently low; and secondly, the existence of a high background of natural and unintentionally produced interferents. Combining the methods to afford both a screening capability and a highly selective detection represents a right strategy to overcome these challenges, as the complexity of CDs components and the drawbacks described for each technique makes that just applying one technique is not enough for a complete analysis of their properties. More specifically, new methods with improved resolution and sensitivity are essential to determine the exact molecular weight of C-dots.

Regarding their biological and biomedical applications, CDs have emerged as an essential innovation in the field, although no CD-based therapeutic product has been approved yet. Just a few clinical trials of nanomaterials were initiated in the past, whether as components of external medical devices or nanosensors, which reflects a remarkable gap between the significant activity, the actual application of CDs in this biological field, and the rate of progress in CDs research. In this sense, safe morphologies and delivery routes of therapeutic CDs are still lacking, as well as the non-specific adsorption of CDs to biological molecules *in vivo* represents a critical drawback to overcome in order to develop a safe profile of CDs, thus avoiding undesired toxic effects or ineffectiveness situations. In this sense, studies focused on *nanopharmacokinetics*, *nanopharmacodynamics* and bioavailability are urgently needed. In the same way, the analysis of environmental interaction of CDs also should be performed in order to reduce it, especially for their biodegradation [139] In conclusion, the great expectancies created around the intense advances and applications of CDs is confirmed. However, further extensive work is still required to get to actual applications, a trend also observed regarding the interaction between C-dots and their use in and for separation techniques.

Conflicts of interest

There are no conflicts of interest to declare.

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Highlights

- C-dots' role in analytical separations is still quite unexplored.
- C-dots based-separation techniques give economic, environmental and applicability advantages.
- Reproducibility in CDs large-scale synthesis and characterizing still remains a challenge.
- Full application of CDs-separation techniques couplings at (bio)analytical field is on its infancy.
- Issues of nanometrology related to CDs use remain to be studied.