CYCLODEXTRIN MODIFIED GRAPHENE QUANTUM DOTS AS A NOVEL ADDITIVE FOR THE SELECTIVE SEPARATION OF BIOACTIVE COMPOUNDS BY CAPILLARY ELECTROPHORESIS

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Acknowledgements

Financial supports from the Spanish Ministry of Economy and Competitiveness (MINECO) and from the Regional Government of Castilla-La Mancha (JCCM) (CTQ2016-78793- P and SBPLY/17/180501/000262, respectively) are gratefully acknowledged. E. Pinilla-Peñalver also acknowledges MINECO for the predoctoral contract BES-2017-080357. M. L. Soriano expresses her gratitude to the European Commission and the Regional Government of Castilla-La Mancha (JCCM) for the funding project SBPLY/17/180501/000333 (PRT program).

32 # Abstract

33 A highly reliable separation and determination of various biologically active compounds
34 was achieved using capillary electrophoresis (CE) based on β-cyclodextrin-
35 functionalized graphene quantum dots (β*cd*-GQDs) as the background electrolyte
36 additive. β*cd*-GQDs improve the separation efficiency between peaks of all analytes. No
37 content of surfactants either organic solvents was needed in the running buffer containing
38 β*cd*-GQDs. Up to eight consecutives' runs were acquired with high precision for the
39 separation of resveratrol, pyridoxine, riboflavin, catechin, ascorbic acid, quercetin,
40 curcumin and even of several of their structural analogues. Baseline separation was
41 achieved within just 13 min as a result of the effective mobility of the analytes along the
42 capillary owing to the differential interaction with the additive. The proposed analytical
43 method displayed a good resolution of peaks for all species selecting two absorption
44 wavelengths in the diode array detector. Detection limits lower than 0.28 μg mL^{-1} were
45 found for all compounds and precision values were in the range of 2.1 − 4.0% in terms of
46 peak area of the analytes. The usefulness of the GQDs-assisted selectivity-enhanced CE
47 method was verified by the analysis of food and dietary supplements. The applicability
48 to such complex matrices and the easy and low-cost GQDs preparation opens the door
49 for routine analyses of food and natural products. The concept of using such dual
50 approach (macromolecules and nanotechnology) has been explored to tackle the
51 separation of various bioactive compounds in nutritional supplements and food.

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59 **Keywords:** Graphitic nanostructure; surface functionalization; molecular receptor;
60 electrophoretic additive; selectivity enhanced; food supplements.

61 **Highlights:**

62 - Design and use of GQDs chemically tailored by *cd* entities at the surface.
63 - First use of β*cd*-GQD conjugates as additive in CE allowing the individual exploitation
64 of the properties of both materials (nanosheet and molecular receptor).
65 - Simultaneous determination of several health-beneficial compounds in nutritional
66 supplements and food.
67 - Those functionalized GQDs greatly improved the separation efficiency and, therefore,
68 the selectivity of seven bioactive compounds by CE.

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85 # Introduction

86 Supramolecular chemistry is the pilar of organized interactions involving molecular
87 organic compounds to be efficient host molecules which many promises in many areas of
88 analytical chemistry, pharmacy and medicine amongst others [1]. The use of cyclodextrin
89 (*cd*) is very versatile and extended for accommodating certain molecules and ions inside
90 its inner hydrophobic cavity forming inclusion complexes, especially in food and
91 pharmacological applications. Advantages of its use relies in its versatility, low toxicity
92 and immunogenicity producing considerably benefits of many hydrophobic or unstable
93 molecules like nutraceuticals and drugs in aqueous media by solubility, permeability and
94 bioavailability enhancements, masking unwanted tastes/odors, or controlling drug
95 release. In particular, from the three types of *cd*, we found that β*cd* cavity is more suitable
96 and easily accessible for many bioactive compounds like flavonoids (eg., flavonols,
97 flavanols, flavones, flavanones), non-flavonoids (eg., stilbenes, hydroxybenzoates),
98 natural diarylheptanoids and vitamins with medium-sized structures in contrast to the
99 insufficient α*cd* inner diameter or the weaker inclusion complexation with the γ*cd* [2].

100 Furthermore, chemically modification of nano-sized materials (NMs) with such cavitands
101 has been explored. In fact, many works explored the advantages of the attachment *cd* onto
102 NMs surface [3] via non-covalent interactions [4-6] whilst others reported covalently
103 functionalization namely by amide coupling reactions, esterifications and etherifications
104 of the hydroxyl groups of the *cd* [7-9].

105 With the appearance of the Analytical Nanoscience and Nanotechnology, the use of
106 several NMs has been improved considerably the separation efficiency of multiples
107 analytes [10]. Although the chromatographic methods achieve high sensitivities, capillary
108 electrophoresis (CE) has also a great potential in separation for its simplicity, minimal of
109 organic waste, short analysis time, high separation efficiency and low cost. In particular,
110 remarkable CE methods had been developed thanks to the use of NMs as additives in the
111 buffer solution, proving an increase in the sensitivity or separation performance of
112 multiple analytes [11, 12]. Amongst the wide variety of nanoparticles exploited (e.g.,
113 metallic, magnetic, carbonaceous, silica), carbon-based NMs have proven to be very
114 promising in separation techniques, reaching excellent selectivity and reproducibility of
115 analysis [13]. However, most carbon allotropes like nanotubes and graphene requires the

116 use of surfactants for their good dispersibility in order to avoid aggregation inside the
117 capillary. Novel hydrophilic carbon based nanodots have attracted considerable attention
118 in analytical separation by virtue of their long-term stability without the need of
119 surfactants or organic solvents, large surface area, and easy chemical modification [14].
120 Graphene quantum dots (GQDs) consist of a water-soluble nanolayer of graphene with
121 quantum confinement that displayed unique photophysical properties, demonstrating
122 great potential as analytical tool in analytical chemistry [15]. For improving GQD
123 properties, many researchers have explored the nanolayers doping with heteroatoms [16]
124 or their functionalization with small molecules [17], like acetic acid for Cardiac Troponin
125 I detection [18]. However, to our knowledge, no reports on GQDs chemically tailored by
126 *cd* cavitands by amidation reaction have been reported until now. The synergistic
127 combination of the ultrasmall graphitic layer with a large adsorption surface and the *cd*
128 as excellent selector of molecules opened many analytical applications.

129 GQDs have been extensively used as sensing probes (optical [19] or electrochemical [20]
130 sensors) while they have been scarcely explored in separation techniques. Note the few
131 analytical methods in which GQDs were explored in CE, finding one example in which
132 such nanographene behaves as a fluorescent enhancer for improving sensitivity of drugs
133 [21] and a second paper describing an improved selectivity in separation of cinnamic
134 derivatives using GQDs as additive [22].

135 Nutraceuticals are food ingredients and dietary supplements containing biologically
136 active compounds that, in addition of having a nutritional function, improving health
137 status, and preventing common diseases and being even part of therapeutic treatments
138 [23]. In view of the growing worldwide usage associated to their health benefits, their
139 efficacy and safety, analytical methods are required for the determination of such food
140 components and those related compounds that nullify the beneficial effects.

141 Herein, a new type of functionalized GQDs with β*cd* was successfully prepared and
142 characterized for the first time. In these NMs, a specific cavitand covalently linked to
143 water-soluble graphene nanolayers was combined. To research the possibilities of β*cd*-
144 GQDs as background electrolyte (BGE) additive in CE, seven health-promoter molecules
145 were efficiently separated under optimum experimental conditions. The developed
146 analytical method was exhaustively validated and applied to the determination of
147 resveratrol (RES), curcumin (CUR), pyridoxine (PYR), riboflavin (RIB), ascorbic acid

148 (ASC), catechin (CAT) and quercetin (QUER) in complex matrices as nutritional
149 supplements and food with satisfactory results. Because in this type of samples, especially
150 in dietary supplements, high concentrations of them are present, we focused on
151 developing an analytical method able to detect, identify and quantify them with high
152 selectivity, being able to distinguish their associated molecules (e.g., isomers). Therefore,
153 it was not so much the desire to achieve very low sensitivities, but rather that the method
154 be very selective and that serves as the basis for the inclusion of new analytes.

155 Experimental section

156 Information related to reagents and materials and instruments is given the Electronic
157 Supplementary Information (ESI.S1).

158 2.1. Synthesis of β-cyclodextrin modified graphene quantum dots

159 Carboxylated GQDs (c-GQDs) were prepared by a simple process previously described
160 by our group [19] and slightly modified. Briefly, uric acid (0.5 g) was subjected to an
161 acidic medium (1 mL of 18.4 M H_2SO_4) under reflux conditions at 200 ºC for 1h. The
162 solution was then cooled down, neutralized and diluted up to 150 mL. The resulting
163 solution was treated with 25 mL of a methanol:acetone mixture (1:4, v/v) and,
164 subsequently, filtered through a 0.22 µm nylon membrane to remove all aggregates. The
165 resulting solution displays a yellow colour.

166 The β*cd*-GQDs was prepared by using a modified procedure previously reported [9].
167 Thus, in a 50 mL two-neck flask containing 57.5 mg of *N*-hydroxysuccinimide (NHS,
168 0.50 mmol), 77 µL of *N,N′*-diisopropylcarbodiimide (DIC, 0.50 mmol) and 11 mL of
169 GQDs (0.9 mg mL^{-1}) were added under an argon flow. The mixture was stirred under
170 inert atmosphere for 15 min. Meanwhile, 80 µL of *N*-tert-butoxycarbonyl-
171 ethylenediamine (Boc-EDA, 0.51 mmol) and 5 µL of triethylamine (TEA, 0.04 mmol)
172 were mixed in vial for 10 min and afterwards added to the previous activated GQD
173 solution. The resultant solution was stirred for 20 h at room temperature under
174 atmospheric conditions of argon. Once the reaction has elapsed, the suspension was
175 filtered with a 0.45-µm nylon membrane in a Millipore system to remove the carbamide
176 formed as a water-insoluble by-product. The Boc-EDA modified GQDs solution was
177 boiled for 15 min to deprotect the amines by releasing the labile Boc group. The resulting
178 product was purified by liquid-liquid extraction for three times with ethyl acetate. The

179 attachment of the *cd* moiety onto GQD surface was achieved by mixing a solution
180 containing the aminated GQDs and 5 µL of TEA (0.04 mmol) with carboxymethyl-β-
181 cyclodextrin (CMβ-*cd*, 0.19 mmol) in presence of DIC (26 mmol) and NHS (0.26 mmol)
182 under inner atmosphere. After 7 h of reaction at room temperature, the suspension was
183 filtered and washed with ethyl acetate thrice. After that, the functionalization degree with
184 amine groups was examined by analysing the oxidized, aminated and *cd*-functionalized
185 GQDs with the Kaiser test.

186 **2.2. Protocol analyses**

187 **2.2.1. Quantification of amines on graphene quantum dots derivatives**

188 Kaiser test [24] analyses of each sample were performed in triplicate to know the content
189 of primary amines onto GQD surface after each stage of the functionalization process.
190 Test tubes containing 100 µL of each GQDs type (carboxylate, amino and *cd* linkers) and
191 100 µL of water as the blank (control) were mixed with 75 µL of the solution containing
192 phenol in ethanol followed by 100 µL of that of KCN in pyridine and, finally, 75 µL of
193 ninhydrin in ethanol. Each test tube was heated for 7 min at 100 °C and finally, 4.8 mL
194 of a mixture of ethanol:water (60:40, v/v) were added. Absorption measurements were
195 recorded at 570 nm and the given values are expressed as µmoles of amino groups per
196 gram of NM.

197 **2.2.2. Electrophoretic separation conditions of bioactive molecules**

198 Separation and quantification of the bioactive compounds were performed in a CE
199 equipment (G1600AX model) from Agilent (Palo Alto, CA, USA) equipped with a diode
200 array detector (DAD). For this, a fused-silica capillary of 42 cm total length (33 cm
201 effective length) and 75 µm of internal diameter (Beckman, Fullerton, CA, USA) was
202 used.

203 Electrophoretic separation was carried out in the positive polarity mode applying a
204 voltage of 10 kV at 25 ºC. Detection was recorded at 278 nm for the separation of RIB,
205 RES, PYR, CAT, ASC and QUER while for CUR was set at 425 nm. Standards and
206 samples were injected by hydrodynamic injection mode for 8 s at 40 mbar.

207 Each day, the capillary was first conditioned by flushing 0.1 M NaOH for 5 min,
208 deionized water for 5 min and, finally, the electrolyte solution for 10 min, applying 2 bars

209 in the external pump. Between runs, the capillary was rinsed sequentially with 0.1 M
210 NaOH (1 min), ultra-pure water (1 min), and fresh running buffer (3 min). Analyses were
211 performed in triplicate and average peak areas were used for quantification. The analytes
212 were identified not only by their migration times, but also by using solutions enriched
213 with the individual compounds.

214 The running buffer consists of an anhydrous sodium tetraborate aqueous solution (40
215 mM, pH 9.5) modified with β*cd*-GQDs at 52 mg L^{-1} as additive in the BGE. Just before
216 use, the buffer was stirred for 30 s and filtered through a 0.45 µm nylon membrane. With
217 the same vials containing the modified BGE, it was possible to ran up to eight analyses;
218 and after that, the BGE vials needed to be replaced by another fresh solution. Although
219 the suspension remains stable for several days, the modified running buffer was prepared
220 daily. Before use, all buffer solutions were filtered through a 0.45 µm nylon membrane.

221 **2.3. Sample preparation**

222 The chosen food supplements were *Vitax* (containing ASC, RIB and PYR, Laboratorios
223 Ferquisa, S.A., Toledo, Spain), *BCAA powder red berries* (containing ASC and PYR,
224 Weider Nutrition, S.L., Madrid, Spain) and *Aquilea Resveratrol* (containing RES and
225 QUER, Laboratorios Uriach, S.L., Barcelona, Spain) which were acquired from local
226 markets from well-known brands and pharmacies. *Vitax* capsules (1.5 g/capsule) were
227 crushed and a portion of 1.2 g was dissolved in 100 mL of an ethanol:water (30%, v/v)
228 mixture. The sample was next stirred by vortex, sonicated for 1h and finally filtered in a
229 pleated filter (a membrane of 125 mm diameter of cotton linters and cellulose fibers with
230 10 – 15 µm of pore size, from FILTER-LAB). The resulting solution was submitted to
231 the electrophoretic procedure for the detection and quantification of ASC, RIB and PYR.
232 However, due to the high amount of ASC in the sample, it was necessary to carry out a
233 1:50 dilution of the previous solution after the filtration step with the same solvent
234 mixture. *BCAA powder* sample was prepared in a similar way, thus, 3.85 g were dissolved
235 in 25 mL of the ethanol:water extractant mixture. This sample was shaken, sonicated for
236 15 min, and filtered afterwards. While for PYR quantification in the extract was directly
237 obtained, in the case of ASC it was necessary to carry out an extra dilution (1:100) prior
238 to injection. *Aquilea Resveratrol* dietary supplement was treated as follows: the powder
239 content of the capsule (0.36 g/capsule) was dissolved in 50 mL of ethanol and sonicated
240 for 30 min. After this time, the suspension was passed through a pleated filter.

241 Subsequently, a dilution (1:25) was prepared fixing a 30% (v/v) ethanol and the
242 nutraceutical content in the samples was determined using the developed electrophoretic
243 methodology.

244 For the preparation of roasted cocoa samples (Ocumare de la Costa, Venezuela), the beans
245 were crushed with a mortar to obtain a uniform powder. A portion of such powder (0.5
246 g) was suspended in 5 mL of a mixture of ethanol:water (30:70, v/v), sonicated for 15
247 min and subsequently filtered through a pleated filter. The remaining cocoa residue was
248 again suspended with 2 mL of the extractant mixture, the suspension was sonicated and
249 filtered again repeating this process 4 more times, so that the total volume of extractant
250 was 13 mL. Each one of the obtained extracts was directly subjected to the CE method to
251 estimate the CAT concentration in the sample. On the other hand, CUR determination
252 was carried out in turmeric spices (India). Turmeric dried powder is mildly aromatic and
253 yellowish orange. A portion of 0.05 g this powder was transferred to a 5 mL volumetric
254 flask, making up its volume with ethanol. The mixture was sonicated for 30 min and
255 subsequently, the suspension obtained was centrifuged for 10 min at 10,000 rpm. Once
256 the undissolved sample portion was decanted at the bottom of the container, the
257 supernatant was diluted (1:12, v/v) with deionized water fixing a 30% final volume of
258 ethanol to maintain its solubility. After that, another extraction from the residual solid was
259 carried out with 5 mL of ethanol, repeating the previous process. Finally, both extracts
260 were separately injected to assess CUR concentration in the sample by the proposed
261 analytical procedure.

262 Results and discussion

263 3.1. Choice of material

264 Many researchers had focused on the use of cyclodextrins in CE as BGE additive for
265 multiple organic molecules separation. There has been particular interest in the use of β*cd*
266 for their inner diameter which is suitable to establish very stable and strong host-guest
267 interactions with a wide variety of organic guests. However, lower solubility for β*cd* (18.5
268 mg mL^{-1}) compared to α- and γ-*cd* is a disadvantage [25]. To solve this inconvenient, a
269 dual approach based on *cd* complexation and nanotechnology has been explored. GQDs
270 were selected by virtue of their fascinating properties and low toxicity, ease of preparation
271 and cheap materials and methods. Water-soluble GQDs is a suitable material to be used
272 in CE, being chosen for easily covalently linked β*cd*. The ensuing system based on *cd*

complexation and nanotechnology displays more selective binding sites to the target analytes and offers a great potential for CE separation technique.

Thus, the surface of GQDs was chemically modified with *cd* for the first time. The first functionalization step relies on reacting the carboxyl groups at the edges of the GQD layers with Boc-EDA and after deprotection of the leaving Boc group, the second step involves the attachment of CMβ-*cd* using amide coupling reactions.

3.2. Synthesis and characterization of β-cyclodextrin-coated graphene quantum dots

High-yielding one-pot synthesis of oxidized GQDs was accomplished by sulfuric acid treatment according to our previous work [19]. Attachment of CMβ-*cd* was achieved by a two-step reaction (9) involving the formation of free amine superficial groups onto the GQDs nanolayers in the presence of monoprotected EDA in a first stage, followed by Boc deprotection and covalent functionalization with the macrocyclic structure via amide coupling reactions for 24 h (**Fig. 1a**).

To confirm the correct functionalization of the GQDs, an exhaustively physic-chemical characterization was performed which indicated that the cavitand structure of β-*cd* is well preserved on the carbon-based nanodots surface after the synthesis process.

The ensuing β*cd*-GQDs were spherical-like structures of 6.2 ± 0.5 nm in diameter (**Fig. 1b-d** shows the narrow distribution and the representative nanolayers), as examined by high-resolution transmission electron microscopy (HR-TEM). The mean hydrodynamic size of 11.7 ± 0.8 nm ($n = 3$) was determined by dynamic light scattering (DLS) technique (**Fig. S1**).

The importance of choosing EDA as the linker to join the cavitand and the GQDs through their carboxylic groups is the easy manner to corroborate the binding by the quantification of free amines at GQD surface. Thus, the surface functionalities of GQDs were assessed by Kaiser test analyses ($n = 3$) and Fourier transform infrared (FTIR) spectroscopy of the three GQD structures containing oxygen, nitrogen groups and β-*cd*. Kaiser test values (**Table S1** and **Fig. S2a**) confirm the incorporation of amine groups in the first functionalization step and the successfully amidation reaction with the CMβ-*cd*.

By FTIR, the peaks assigned to the β*cd*-GQD structure appeared at 3307, 2920, 1670, 1559, 1429 and 1367 cm^{-1} which are characteristic of hydrogen-bonded O-H stretching,

303 C-H stretching, C=O and C=C stretching, C-H and O-CH in-plane bending, and C-H
304 deformation, respectively (**Fig. S3**). Peaks from the cavitand, which were slightly shifted
305 from the free one (1637, 1157, 1026, 672 cm^{-1}), appeared at 1644, 1144, 1029 and 640
306 cm^{-1}. In comparison to the oxidized GQDs depicted in **Fig. S3**, it is clear that β-*cd* was
307 successfully attached to the GQD surface.

308 Optical characterization of these nanodots was achieved by UV-vis spectrophotometry
309 and fluorescence techniques. A broad absorbance band centered at 365 nm was found for
310 both c-GQDs and β*cd*-GQDs (**Fig. S2b**) and their maximum emission (by exciting at 365
311 nm) appeared at 443 and 457 nm for raw and functionalized ones (see **Fig. S4**) as a result
312 of a variation of the non-radiative recombination of their electronic transitions [26].

313 Thermal gravimetric analysis (TGA) is one of the useful techniques to characterize
314 functionalized NMs after exposing them to a gradually increasing temperature under inert
315 atmosphere for quantifying the respective weight losses. Then, thermal stabilities of c-
316 GQD and β*cd*-GQD were examined by TGA under nitrogen flow to check the functional
317 groups incorporated to the surface. After stabilization of the temperature at 100 ºC for 20
318 min to remove residual water, TGA plots depicted in **Fig. S5** show two major steps of
319 weight loss, being higher from the cavitand modified GQDs (22.3 and 33.8%) versus the
320 c-GQDs (11.7 and 30.1%). Furthermore, the second derivative displays another minor
321 step of weight loss (27.4%) at temperature above 400 ºC. It is expected that this gradual
322 decrease in the TGA curve at $T^a \geq 400$ ºC is due to the carbon burning from the graphitic
323 layers. Functionalized GQDs with cyclodextrin is then less stable and decomposes at
324 lower temperatures than the carboxylate one.

325 Batch to batch reproducibility of β*cd*-GQD was also evaluated in terms of emission
326 intensities of the resulting GQDs, resulting in 94.9% with a standard deviation of 3.1%.

327 **3.3. Separation of the bioactive compounds by capillary electrophoresis**

328 To avoid the extensive use of harmful additives that causes water polluting and endangers
329 aquatic life and human health, in the developed method neither organic solvents nor
330 organic modifiers were used. Additionally, their use in high quantities tends to have
331 general problems of high currents in CE. Instead, it has been explored the separation of
332 seven analytes in the absence and the presence of non-toxic GQDs with diverse surface
333 as nanoscopic modifiers. The advantage of using water-soluble GQDs instead of the

334 commonly used carbon nanoparticles (graphene, carbon nanotubes, nanodiamonds and
335 fullerenes) lies in that not surfactants are required to stabilize them [27, 28], and thus, no
336 bubble formation may cause an interruption in the separation process.

337 The influence of key parameters affecting the sensitivity and selectivity of the seven
338 chosen bioactive compounds (RIB, RES, PYR, CAT, ASC, QUER and CUR) was
339 evaluated to develop an effective separation method. **Table S2** shows the molecular
340 structure of the analytes and their corresponding pKa values.

341 **3.3.1. Effects of pH and concentration of the running buffer**

342 The pH of BGE is an important parameter that affects the separation of the study analytes
343 since whether their ionization is dependent on such pH. Initially, it was evaluated the
344 influence of the pH using a set of phosphate or borate salts buffer solutions at pH 8.5 −
345 10.0 with a concentration of 40 mmol L^{-1} of the corresponding salt. This pH range was
346 selected according to the pKa of the analytes, being negatively charged. As shown in **Fig.
347 S6a**, the BGE at pH 8.5 allows the baseline separation of only RIB, RES, ASC and QUER
348 with good resolution, even though their peaks are not very symmetric, while PYR and
349 CAT could not be separated, appearing as a single peak. Curiously, by increasing the pH
350 up 9.0, the latter peak began to unfold, although no separation was achieved.
351 Unfortunately, the migration times of RIB and RES were much closer to each other than
352 before, turning both peaks overlap. At pH 9.5, all peaks were visualized in the
353 electropherogram although no baseline separation was observed between the first eluting
354 analytes. More basic pH difficult the separation of the analytes and greatly increases their
355 migration times. Thus, borate buffer solutions fixed at pH 9.5 were selected for further
356 studies.

357 Secondly, the effect of ionic strength of the BGE was also investigated. The optimal ionic
358 strength of the electrolyte should be a balance between a good peak resolution and a
359 current not too high to minimize background noise. The effect of borate buffer
360 concentration of BGE on the separation of the nutraceutical compounds was evaluated at
361 concentrations ranging 10 − 50 mM at pH 9.5. **Fig. S6b** shows that at increasing
362 concentrations borate salt, migration times are longer and the separation between all of
363 them was improved, although no baseline separation was achieved for all analytes in none
364 of the cases. As can be seen from results, good resolution of CAT and ASC occurred at
365 above 30 mM. In short, the resolution for all analytes improved at higher ionic strength

366 up to 40 mM, while over this concentration no further improvement was observed,
367 negatively involving higher currents and longer migration times. The latter is probably
368 due to the fact that increasing the concentration of BGE leads to an increase in ionic
369 strength, which results in a reduction in the thickness of the electrical double layer and a
370 decrease in electroosmotic fluid (EOF). This leads to an increase in the migration time of
371 the compounds and, therefore, to a slight improvement in the separation between peaks.

372 The best conditions found for the separation of most analytes were between 30 and 40
373 mM, therefore both concentrations were selected for subsequent studies. However, even
374 under the selected separation conditions, specifically RIB, RES and PYR could not be
375 baseline separated as shown in **Fig. 6S.b**(*III,IV*). Therefore, the use of additives to the
376 running buffer to enhance the separation are needed.

377 **3.3.2. Effect of GQDs types as additives in the running buffer**

378 The addition of c-GQDs and β*cd* functionalized GQDs as additives in the BGE was
379 examined at diverse concentrations.

380 Initially, c-GQDs were added in the BGE at concentrations from 0.25 to 1.00 mg mL^{-1}
381 using a set of 30 and 40 mM of borate buffer solutions at pH 9.5. Interestingly, at
382 increased c-GQDs concentrations, the resolution obtained between RIB, RES and PYR
383 peaks improved, although peak deformity was found. The best resolution was found using
384 0.75 mg mL^{-1} of c-GQDs. The peak broadening problem observed, and high currents
385 generated could perhaps be due to the heat emission within the capillary by the Joule
386 effect when using such high concentrations of NM.

387 Next, β*cd*-capped GQDs were designed and added as additive to the BGE to differently
388 influence the analytes to achieve the separation of the chosen compounds as a pseudo-
389 stationary phase. In this case, the added amounts of β*cd*-GQDs into both 30- and 40-mM
390 borate buffer solutions (at pH 9.5) were in the range of 35 − 86 μg mL^{-1} (**Fig. 2**). After
391 the addition of β*cd*-coated GQDs to the BGE, an improved separation between all the
392 nutraceutical compounds was finally achieved as a result of the interactions of β*cd*-
393 functionalized GQDs with the analytes. The best results in terms of peak resolution were
394 obtained with 68 μg mL^{-1} of the NM when using 30 mM borate buffer, although higher
395 concentrations induced a broadening of peaks. In the case of 40 mM of borate salt, an
396 improved resolution was found at 52 μg mL^{-1} of β*cd*-GQDs. In view of the peak resolution

397 found for both concentrations, it was selected 40 mM borate aqueous solution as it
398 requires less concentration of the additive. So, it was chosen the 40 mM buffer solution
399 modified with 52 µg mL^{-1} β*cd*-GQDs as running buffer. As previously mentioned, an
400 increase in ionic strength induces longer migration times which could provide a greater
401 interaction between the analytes and the stationary phase. In fact, there is a better baseline
402 separation of the analytes when the buffer concentration is 40 mM borate instead of 30
403 mM. **Fig. S9** summarizes the best peak resolution attained in the absence and presence of
404 β*cd*-GQDs (52 µg mL^{-1}). The improvement of the separation efficiency is evident by
405 adding β*cd*-GQDs to the BGE. In the presence of β*cd*-GQDs, the resolution between
406 peaks of RIB, RES, PYR and CAT (the closest ones without the additive) that were poor,
407 improved up to a threefold achieving a good resolution between them (**Table S3b**).

408 It was demonstrated that the migration time of most of the analysed compounds increased
409 at diverse extend in the presence of GQDs functionalized with cyclodextrins, resulting in
410 good resolution between some peaks. This fact can be attributed to interactions between
411 the analytes and the NM, which may alter the EOF by decreasing its mobility or due to
412 an increase in the electrophoretic mobilities of the analytes. To demonstrate what causes
413 the observed behaviour, experiments were carried out to evaluate the effect of NM
414 concentration on the mobility of the EOF when the other conditions remained constant.
415 Acetone was chosen as a neutral EOF marker, and the mentioned mobility was calculated
416 as follows (equation 1):

417 $$\mu_{EOF} = \frac{l \cdot L}{t_m \cdot V} \qquad Eq\ (1)$$

418 where μ_{EOF} corresponds to the EOF mobility; l and L are the effective (33 cm) and total
419 (42 cm) length of the capillary, respectively; t_m is the migration time taken by acetone to
420 migrate from the inlet to the detection window and V is the voltage applied across the
421 capillary column. As depicted in **Fig. S7**, the EOF mobility remains practically constant
422 in the concentration range of the essayed NM. It is indicated that at pH 9.5 both β*cd*-
423 GQDs and capillary wall exhibit negative charges, and therefore, due to repulsions
424 between charges, nanoparticles are not adsorbed on the capillary wall. Thus, the presence
425 of β*cd*-GQDs inside the capillary reduces the electrophoretic mobility of the target
426 analytes, increasing their migration time.

427 **3.3.3. Influence of voltage and injection time and pressure**

428 Electropherograms depicted in **Fig. S8** show a reduction in the migration times of all
429 analytes when applied higher voltage as expected. On the other hand, sensitivity of signals
430 resulted in an increase at longer times of injection, although problems occurred related to
431 the broadening and even overlapping of peaks. Information regarding the influence in
432 voltage and injection time and pressure parameters are detailed in ESI.S2. The optimized
433 conditions were as follows: 8 s of injection time, 40 mbar of pressure and 10 kV of applied
434 voltage.

435 3.4. Interaction of bioactive compounds with β-cyclodextrin modified graphene 436 quantum dots

437 The sufficient deep cavity of the β*cd* is enough to accommodate most of the biologically
438 active molecules chosen. In addition, it was found that the water-soluble β*cd*-GQD
439 behaves as an excellent additive avoiding the use of surfactants for the binding and
440 separation of organic compounds that contains a variety of organic moieties like five-
441 (ASC) and six-member rings (PYR, RES and CUR) and more space-filling ones like
442 flavanols (QUER and CAT).

443 According to the structure of the analytes, their interaction with the additive is different.
444 Thus, it is revealed by **Fig. S9** that apparent mobility of most of organic compounds
445 increases at diverse extend in the presence of β*cd*-GQD. The first three unseparated peaks
446 in the absence of the additive were RIB, RES and PYR. Their separation is accomplished
447 with the use of β*cd*-GQDs that interacts differently with the target analytes. In fact, it is
448 reported that RIB interacts with β*cd* by *out-of-ring* RIB-*cd* interactions [29], which may
449 suggest herein that RIB interacts weakly with the GQD layer and does not enter into the
450 macrocycle. In contrast, the size and shape of the RES is adequate for the strong binding
451 with the inner part of the cavity, increasing their electrophoretic mobility and allowing
452 the perfect separation of RES and RIB by CE. PYR is also a small organic guest capable
453 of forming stable inclusion complexes [30] attributed to hydrogen bonds with the β*cd* of
454 GQDs, increasing its migration time for a efficiently separation from RES and RIB.
455 Similar effect occurred with ASC that should be encapsulated inside the β*cd*, attached
456 into GQDs, as others reported [31] while in the case of QUER and CAT the interactions
457 with the inner part of the cavity is thanks to their catechol group [32, 33]. In fact, all
458 analytes interact with the β*cd*-GQD in different extend allowing a complete separation.

459 3.5. Analytical performance characteristics

460 The analytical performance of the proposed electrophoretic method was studied in order
461 to know its usefulness for the quantitative determination of RIB, RES, PYR, CAT, ASC,
462 QUER and CUR. Several analytical features were evaluated under the optimized
463 experimental conditions. The analysed nutraceutical compounds were adequately
464 separated and quantified in less than 13 min.

465 External calibration curves of the compounds were constructed using peak areas as a
466 function of their concentration and injecting each of the standard solutions in triplicate
467 from 0.1 to 35.0 µg mL^{-1}. A good linear relationship for all analytes was observed in the
468 0.5 – 25.0 µg mL^{-1} range, except for QUER which was between 0.3 and 13.0 µg mL^{-1} and
469 for CUR from 0.8 to 30 µg mL^{-1}. Good linearity was obtained for all cases with
470 determination coefficients ranging between 0.994 and 0.999.

471 The precision of the proposed method was assessed in terms of repeatability and
472 reproducibility expressed as relative standard deviation (RSD). The repeatability of the
473 electrophoretic procedure was evaluated both as function of peak area and migration time.
474 For that, five independent injections of standard solutions were carried out sequentially
475 at 7 and 10 µg mL^{-1} for QUER and the rest of analytes, respectively. RSD values (*intra-*
476 *day* conditions) were between 2.1% and 4.0% in terms of peak area and from 1.5% to
477 2.2% for migration time. The high repeatability obtained indicates the stability of the
478 analytical approach and, therefore, the reliability of the analytical method. To test the
479 reproducibility of the measurements, the same experience was repeated over 3 days,
480 obtaining RSD values (n = 15) lower than 4.7% and 2.8% for peak area and migration
481 time, respectively.
482 The limits of detection and quantification (LOD and LOQ) for each analyte were also
483 calculated. These statistical parameters are defined as the analyte concentration that
484 provides a signal equivalent to the blank signal plus three and ten times its standard
485 deviation, respectively. LOD values achieved ranged from 0.1 to 0.3 µg mL^{-1} while those
486 of LOQ were between 0.3 and 0.8 µg mL^{-1}. Once the sensitivity of the method was
487 estimated, LOQ values obtained were experimentally corroborated independently by
488 analysing five standards prepared at the previously estimated concentration for each
489 analyte. **Table 1** summarizes the analytical parameters obtained for the analysed analytes
490 using β*cd*-GQDs as additives in the BGE.

491 The analytical features of the proposed separation method were assessed by setting the
492 diode array detector at the maximum absorption of each of the analytes to achieve the
493 highest sensitivity.

494 **Table 2** summarizes the main analytical characteristics of different CE separation
495 methods of bioactive compounds in food and pharmaceutical samples [11, 34-38].
496 Despite the high sensitivity of liquid chromatography technique (**Table S4)**, the proposed
497 electrophoretic method reaches lower LOD values for some compounds in comparison to
498 other works that determined simultaneously coincident analytes by CE, in particular for
499 RIB and ASC [11, 34, 35], PYR [11, 35], RES [37] and QUER [38]. Furthermore, it is
500 worth noting the short analysis time to achieve the separation of all seven compounds.
501 This suggests its use in effective and reliable routine assays for the simultaneous
502 determination of a wide variety of compounds in nutritional supplements and food.

503 **3.6. Analytical application**

504 The nutraceutical content of the studied compounds was determined in different dietary
505 supplements (*BBC powder*, *Vitax* and *Aquilea Resveratrol*) as well as in other food
506 samples such as cocoa beans or turmeric spices containing some of the analytes of interest
507 to demonstrate the potential applicability of the proposed analytical method.
508 Each sample was prepared according to the procedure described in "*Sample preparation*"
509 Section and submitted to the analytical methodology detailed in "*Electrophoretic*
510 *separation conditions of bioactive molecules*". All samples were analysed in triplicate.
511 In case of dietary supplements, no interferences that could influence the peaks of the
512 studied analytes were observed. The concentration of nutraceutical compounds which
513 was estimated by external calibration, turned out to be very similar to that declared in the
514 product labels. These results are summarized in **Table 3** and their electrophoretic profiles
515 are shown in **Fig. S10a-c**.
516 For cocoa sample, there was no reference either information in the product regarding the
517 amount of catechin contained, occurring the same for the turmeric spices concerning its
518 curcumin content. Therefore, standard additions were performed to compare the results
519 obtained by another type of calibration and to evaluate the matrix effect of these samples,
520 since a wide variety of compounds present that absorb at the recording wavelengths.
521 Cocoa beans samples were fortified at four concentration levels of CAT standard solution
522 between 0.052 and 0.260 mg g^{-1} while turmeric spices were spiked with increasing

523 concentrations of CUR in the 3 − 12 mg g^{-1} range. The obtained concentrations of CAT
524 for cocoa sample were 0.091 ± 0.004 mg g^{-1} (n = 3) and 0.100 ± 0.005 mg g^{-1} (n = 3) by
525 external and standard addition calibration, respectively. For the case of turmeric sample,
526 a CUR concentration of 23.7 ± 0.7 mg g^{-1} (n = 3) was obtained by external calibration
527 and by the standard addition method was of 24.9 ± 1.4 mg g^{-1} (n = 3). The application of
528 the *Student t* test for a confidence level of 95% showed no significant differences in the
529 concentrations obtained by both calibration methods for cocoa and turmeric samples.
530 Once standard additions were performed, recovery values in the range of 98.0 – 103.8%
531 were found for cocoa sample and between 97.1 and 101.8% for turmeric spices as can be
532 seen in **Table 4**. The electropherograms corresponding to the cocoa beans and turmeric
533 samples are depicted in **Fig. S10d-e**.
534

535 In addition, to verify the applicability of the proposed electrophoretic method, *Aquilea*
536 *Resveratrol* sample, which endogenously contains RES and QUER as previously
537 demonstrated, was doped with the other analytes, and analysed by the proposed method.
538 In this way, the method can be applied to a sample that contains all the selected
539 nutraceutical compounds. As shown in **Fig. 3**, it is clear that a very good baseline
540 separation was achieved for all seven analytes when the NM was used as an additive.
541 Furthermore, other compounds from the matrix did not interfere with the peaks of the
542 target analytes.

543 Finally, to demonstrate the selectivity of the proposed separation method, the presence of
544 other compounds associated with the target analytes was also evaluated. One the one
545 hand, two additional curcuminoids, specifically desmethoxycurcumin (DMCUR) and
546 bisdemethoxycurcumin (BDMCUR), were added to a solution containing all seven
547 nutraceuticals. On the other hand, the mentioned mixture was subjected to a 365-nm of
548 light irradiation for 3 min to convert RES into its isomeric product, *cis*-RES. In
549 preliminary studies, it was observed in the mixture how RES was turned into the *cis*-
550 isomer when irradiated with UV light, as found in literature [39]. Although the similar
551 structure between the three selected curcuminoids and the two resveratrol isomers, a good
552 resolution for all of them was achieved applying the proposed methodology (**Fig. S11**).
553 With the developed separation method, all target analytes and some of their derivatives
554 were well-identified demonstrating its suitability. These results proved the applicability

of the analytical procedure for routine analysis of a wide variety of samples, quality control or their characterization.

4. Conclusions

A novel type of β*cd* modified nanoparticle was designed and prepared showing unique properties, combining those typical from water-soluble GQDs as support in addition to the advantages of natural cyclodextrins to form inclusion complexes. β*cd*-GQDs behaves as an interesting CE additive for the efficiently separation of seven health-promoter compounds (polyphenols and vitamins), decreasing their electrophoretic mobility as a result of their interaction with the *cd* functionalized GQDs. It is expected that the interaction with RIB is mainly with the graphene layer while the rest of the analytes may establish a specific host-guest interaction with the linked macrocycle in different extend depending on the size and flexibility of the structure, which is the driven key to effectively separate the bioactive molecules. Thus, a simple and selective method was developed and thoroughly validated, obtaining satisfactory results that made it suitable and feasible for the routine analysis of a wide variety of compounds of biological interest. Analyses of food samples and dietary supplements were performed for the target analytes, verifying the accuracy of the results. The most remarkable aspect of this novel CE procedure is that no surfactants either organic solvents were required, neither tedious sample treatment for the simultaneous detection of such bioactive compounds, serving as the basis of screening multiple analytes in complex matrices like food.

583 ## Declaration of interest

584 The authors have no other relevant affiliations or financial involvement with any
585 organization or entity with financial interest in or financial conflict with the subject matter
586 or materials discussed in the manuscript apart from those disclosed.

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606 # Figures caption

607 **Graphical abstract** Schematic illustration of the electrophoretic separation of the
608 bioactive molecules in the capillary which is filled with the running solution without (top)
609 and with β*cd*-GQDs (bottom). The fused silica capillary with negatively ionizable silanol
610 groups at the wall. The voltage is applied at positive polarity at the outlet. *R*: riboflavin;
611 *r*: resveratrol; *P*: pyridoxine; *C*: catechin; *c*: curcumin; *A*: ascorbic acid; *Q*: quercetin

612 **Fig. 1** Synthetic route for the functionalization of GDQs with β*cd* (a). Size distribution
613 (b) and representative TEM micrographs of the ensuing β*cd*-GQDs (c-d)

614 **Fig. 2** Electropherograms found at 30 mM (a) and 40 mM (b) of borate buffer in the absence
615 (*I*) and presence (*II*-*V*) of increasing concentrations of β*cd*-GQDs in the BGE. The
616 concentrations of the NM are 43 (a.*II*), 52 (a.*III*), 68 (a.*IV*) and 86 μg mL^{-1} (a.*V*) in the left
617 graph, and 35 (b.*II*), 43 (b.*III*), 52 (b.*IV*) and 68 μg mL^{-1} (b.*V*) in the right one. Riboflavin
618 (1), resveratrol (2), pyridoxine (3), catechin (4), ascorbic acid (5) and quercetin (6) are the
619 target analytes

620 **Fig. 3** Electrophoretic profiles corresponding to *Aquilea Resveratrol* sample containing
621 resveratrol (2) and quercetin (6) and fortified with riboflavin (1), pyridoxine (3), catechin
622 (4), ascorbic acid (5) and curcumin (7). Signals recorded at 278 (a) and 425 nm (b) in the
623 absence (*I*) and presence (*II*) of β*cd*-GQDs

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