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A selective spectrofluorimetric method for carbendazim determination in oranges involving inclusion-complex formation with cucurbit[7]uril

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ABSTRACT

The increase in fluorescence intensity with respect to carbendazim that occurs as a result of supramolecular-complex formation between carbendazim and cucurbit[7]uril has been studied. This host–guest interaction has been employed to develop a sensitive and selective method for benzoimidazole-type pesticide determination in fruit samples. The association constant and stoichiometry of the complex formed are reported herein, and the influence of experimental variables, such as the pH or ionic strength of the solution, on complex formation and the presence of interfering substances is also discussed. Under the optimal conditions found, the developed method allows the detection of carbendazim at a 5.0×10^{-9} M level. To test the method, matrix solid phase dispersion was employed as a sample preparation method for carbendazim determination in orange samples with an RSD (%) ($n = 3$) value of 5%. The LOD and LOQ values calculated for real samples were 0.10 and 0.52 mg/kg, respectively, thus showing that the proposed method is sensitive enough to meet legal requirements.

1. Introduction

Cucurbit[*n*]urils (CB[*n*], $n = 5$–8, 10; Fig. 1a), a family of macrocyclic host molecules containing glycoluril units linked by a pair of methylene groups [1], were first reported by Behrend et al. [2], although it was another 70 years before Freeman et al. [3] reported the molecular structure of CB[6]. As numerous new methods for increasing the yields of the congeners ($n = 5$, 7, 8, 10) in comparison with the major product CB[6] have since been reported [4,5], cucurbiturils have attracted increasing interest in the field of supramolecular chemistry.

The name "cucurbituril" is derived from the pumpkin-like shape of these molecules (cucurbitacea family). Fig. 1b shows the general structure of these macrocyclic receptors, which possess a hydrophobic inner cavity and two restrictive portals lined with ureido carbonyl groups. These characteristics mean that CBs are able to form remarkably stable complexes with a variety of guest molecules in aqueous solution as, in addition to the hydrophobic interactions within the cavity, the carbonyl groups are capable of stabilizing the host–guest complex through hydrogen bonding, ion–dipole or dipole–dipole interactions [6]. Although the inner cavity sizes of CB[6], CB[7] and CB[8] are comparable to those of α-, β- and γ-cyclodextrins (CDs), there are remarkable differences between both receptor families. Thus, CDs are natural compounds resulting from action of the enzyme cyclodextrinase on starch, whereas CBs are synthetic products. Likewise, CBs present an equatorial symmetric plane that leads to two identical open portals, whereas CDs are chiral receptors with a toroidal shape and therefore two different uncharged portals. This means that the binding properties of CDs are quite different to those of CBs, which interact with guest molecules via different intermolecular forces, namely hydrophobic interactions with the hydrophobic inner cavities (like CDs) and ion–dipole interactions between negative CB portals. This means that higher association constants are found for CBs than for CDs with the same guest [7]. CB[6] is extremely insoluble in common solvents except for highly acidic aqueous solutions or aqueous solutions containing alkali metal salts. All CB homologues except CB[5] and CB[7], which are moderately soluble in water ($2–3 \times 10^{-2}$ M, similar to β-cyclodextrin) [8], present a similar behavior. The water solubility of CB[7] and its intermediate size make it particularly attractive for a wide range of applications. In particular, CB[7] has a significant beneficial effect on fluorescent dyes by increasing their fluorescence intensity, solubilization and deaggregation, enhancing their photostability and providing some protection against fluorescence quenchers [9,10]. This strong affinity of CB[6] and CB[7] for organic dye molecules makes them suitable for the treatment of effluents from the dye industry [11]. Although spectroscopic methods are preferred to study CB-complex formation, electrochemical techniques are also useful tools to this end. Kaifer et al., for instance, have used electrochemical methods to show that CB[7] binds preferentially to charged guests (MV^{2+} vs. MV^{0}) whereas CDs prefer neutral molecules as

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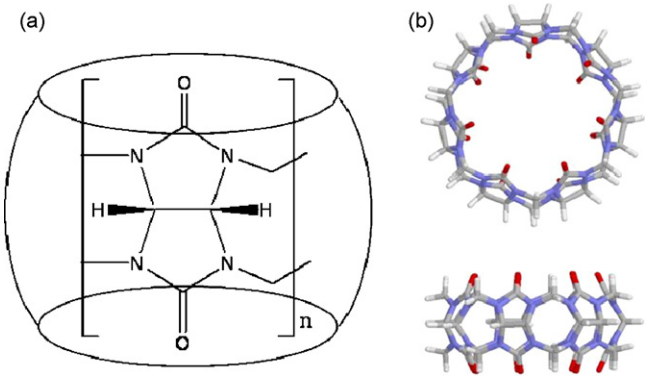

Fig. 1. Cucurbit[*n*]urils structure.

guests [12,13]. Other guests such as imidazolium-based ionic liquids [14], histamine H2 [6] or benzimidazole fungicides [15] have also been studied for CB[*n*] inclusion.

Although there have been an increasing number of papers related to different CB[*n*]s host–guest interactions with a wide range of compounds over the last few years [16], their potential analytical applications (i.e. improving the selectivity and sensitivity of analytical methods) remain relatively unexplored. Saleh and Rawashdeh [15], for example, reported the 1:1 complex formation between carbendazim (CBZ) and CB[6] and calculated the association constant, along with other thermodynamic parameters, in a neutral medium (0.2 M Na_2SO_4). Herein we report a sensitive and selective analytical procedure for determination of a benzimidazole-type fungicide (CBZ) in real samples that involves CB[7] complex formation. To the best of our knowledge, this is the first report of such a procedure.

Benzimidazole-based fungicides and pesticides have broad-spectrum efficacy for the control of a great number of insect species on different crops, especially cereals, fruits and stored fruits, wines and mushrooms [17]. Despite the benefits derived from the use of benzimidazole pesticides in food production, they are easily introduced into the environment and their toxicity (teratogenicity, congenital malformations, etc.) [18,19] has forced the European Union to include these compounds in their list of priority pollutants and to establish maximum residue limits for individual pesticides in different samples [20]. Although different analytical methods have been reported for determination of these pesticides, the development of new, rapid, simple, sensitive and selective methodologies for pesticide residue analysis is still required.

2. Experimental

2.1. Reagents

Cucurbit[6]uril and -[7]uril were obtained from Sigma-Aldrich Chemical Co. (St. Louis, USA). Stock solutions were prepared in 0.2 M NaCl at a concentration of 6.02×10^{-4} M and used for further dilution in the supporting electrolyte. A pure standard of the target compound carbendazim (99%) was purchased from Sigma–Aldrich Chemical Co. (St. Louis, USA), a working stock solution prepared in dimethyl formamide at a concentration level of 7.00×10^{-3} M and this solution used for further dilution and spiking of the samples. Standard solutions were prepared daily and stored at 4 °C in the dark. Thiabendazole (99.8%), diuron (99.5%), thiamethoxam (99.7%) and benomyl (99.4%) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA).

C18 (55–105 μm) was purchased from Waters (Milford, USA) and washed sea-sand (0.25–0.30 mm) from Panreac (Barcelona, Spain).

All reagents used were of analytical reagent grade. Solvents were purchased from Scharlau (Barcelona, Spain), and Milli-Q water was purified with a Milli Ro Milli Q Plus 185 apparatus from Millipore (Waters, Milford, USA).

2.2. Apparatus

Fluorescence measurements were carried out with a HITACHI F-7000 Fluorescence Spectrophotometer supplied by Genesys Instrumentation (Madrid, Spain).

A grinder (Moulinex 1,2,3; Madrid, Spain) was used for sample preparation, and a Vac Elut system (Micron Analitica, Madrid, Spain) was used as vacuum-manifold system for sample preparation. A Techne DRIBLOCK DB 20 sample concentrator equipped with temperature control and N_2 flow (Genesys Instrumentation, Madrid, Spain) was employed to concentrate the sample extracts collected.

Mass spectrometry experiments were performed with a REFLEX III Mass Spectrometer (Bruker BioSciences Española S.A., Madrid, Spain).

2.3. Procedure

2.3.1. Fluorescence measurements

Emission spectra for both CBZ solutions and complexes were recorded employing 1 cm^2 quartz cuvettes at an excitation wavelength of 285 nm. For CBZ determination, the fluorescence CBZ-CB[7] intensity was monitored at 302 nm in 10^{-4} M acetate buffer, pH 4.0, at room temperature.

2.3.2. Sample preparation

Oranges were purchased from a local market, peeled, and the peel dried at 100 °C after being chopped and homogenized. The matrix solid phase dispersion (MSPD) sample preparation method was carried out as follows: an accurately weighed 0.5 g sample of the dried untreated peel (corresponding to 1.64 g of wet sample) was placed in a porcelain mortar and mixed with 0.5 g of C18 and 0.2 g of sea-sand by gentle grinding with a pestle. This treatment allowed disruption of the sample and its dispersion on the sorbent's surface. The homogenous dried mixture was then packed into a glass dispensable extraction cartridge (0.8 cm × 6.5 cm; Varian, Spain) equipped with a propylene frit on the bottom and installed in the vacuum-manifold system. The sample was extracted with 10 mL of dichloromethane, which was allowed to elute dropwise by applying a slight vacuum. The eluent was concentrated to dryness under a gentle nitrogen flow and the residue dissolved in 200 μL of methanol diluted to 2.0 mL in 10^{-4} M acetate buffer solution after CBZ addition. Finally, it was filtered through a 0.45-μm disposable syringe filter before fluorescence measurements. Unless otherwise specified, experiments were carried out in triplicate and in a fume hood to prevent any possible contamination.

3. Results and discussion

3.1. Carbendazim complexation by CB[n]

From an analytical perspective, the increase of fluorescence resulting from the host–guest properties of CBs opens up a wide research field concerning the development of sensitive analytical methodologies. To develop a sensitive and selective analytical method for CBZ determination, the use of different CBs (CB[6] and CB[7]) was evaluated to determine the best experimental conditions. Formation of both the respective inclusion complexes was confirmed by MALDI-TOF mass spectrometry Thus, the spectrum of CBZ-CB[6] was found to contain a peak at *m*/*z* 1188.4 corresponding to CBZ^+-CB[6], thereby supporting the formation of

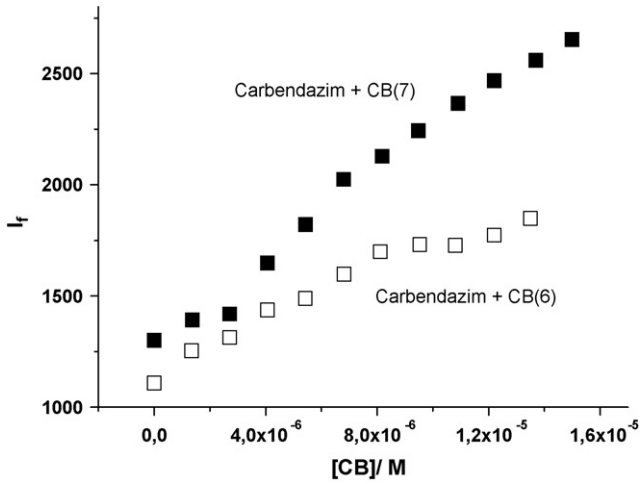

Fig. 2. Variation of fluorescence intensity with increasing CB[6] and CB[7] concentrations. 0.2 M acetate buffer, pH 4.0; λ_{exc} = 298 nm; λ_{em} = 305 nm.

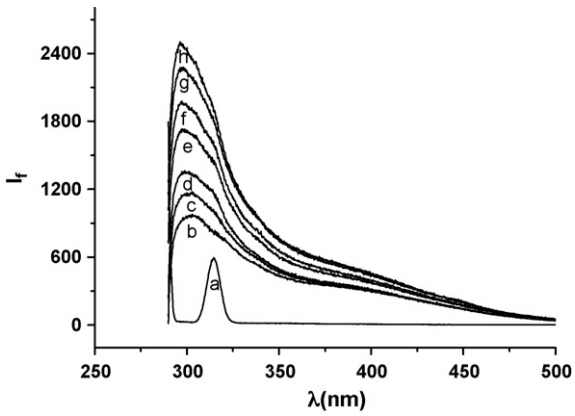

Fig. 3. CBZ (2.7×10^{-6} M) fluorescence enhancement with successive CB[7] additions. (a) 0.2 M acetate buffer, pH 4; (b) 2.7×10^{-6} M CBZ; (c) $+1.4 \times 10^{-6}$ M CB[7]; (d) $+2.7 \times 10^{-6}$ M CB[7]; (e) $+4.1 \times 10^{-6}$ M CB[7]; (f) $+5.4 \times 10^{-6}$ M CB[7]; (g) $+6.8 \times 10^{-6}$ M CB[7]; (h) $+8.2 \times 10^{-6}$ M CB[7].

a 1:1 host–guest complex, as reported previously by Saleh and Rawashdeh [15]. Likewise, the mass spectrum of CBZ-CB[7] also showed a simple signal at m/z 1354 corresponding to the 1:1 complex CBZ^+-CB[7]. As expected, both spectra clearly indicated the formation of ionic complexes between CB[n] and Na^+ ion from the matrix (at m/z 1019.3 for CB[6]–Na^+ and m/z 1185.3 for CB[7]–Na^+). These results show that both CB[6] or CB[7] can be employed as hosts for CBZ. To select between these two possibilities, fluorescence spectra of solutions with increasing $CB[n]_{n=6,7}$/CBZ ratios were recorded. As with CB[6], CBZ-CB[7] complex formation leads to an enhancement of the fluorescence intensity with no change in the maximum emission wavelength (λ_{em}) with respect to CBZ. The change observed in the slopes in the I_f/CB[n] plot recorded at acidic pH (see Fig. 2) suggests different stoichiometric complex formation with increasing CB[n] amounts. The association constants for each complex were calculated from these experiments using the Benesi–Hildebrand method. The values obtained for CBZ-CB[6] and CBZ-CB[7] were $K_1 = 7.3 \times 10^4\ M^{-1}$, $K_2 = 1.0 \times 10^6\ M^{-1}$ and $K_1 = 6.6 \times 10^3\ M^{-1}$, $K_2 = 7.5 \times 10^6\ M^{-1}$ respectively. These values are much higher than those reported previously for CBZ-CD complexes [21] or for CBZ-CB[6] under different experimental conditions [15]. Although the first association constant is somewhat higher for CB[6] than for CB[7], the increase in fluorescence intensity with respect to the CBZ signal produced upon CB[7]-CBZ complex formation was, in all cases, much higher than the variation observed upon CB[6] complex formation, therefore CB[7] was chosen for further studies.

3.2. CBZ-CB[7] complex formation

3.2.1. Influence of pH and buffer concentration

Carbendazim presents two pKs. Thus, at a pH below 4.5 (pK_{a1}) the guanidinium group is protonated whereas above pH 10.6 (pK_{a2}) the carbendazim carbamide group remains negative; the neutral form of the analyte is present at intermediate pH values [22]. It was therefore decided to study the influence of solution pH on CB[7]-CBZ complex formation, fluorescence intensity and stoichiometry by varying the pH of the medium in the range 1–12. Fluorescence spectra of CBZ and CBZ-CB[7] solutions with increasing CB[7] ratios were recorded in all cases. The CBZ concentration was kept constant at 2.7×10^{-6} M.

As expected, the largest changes in the fluorescence signal were observed at pH's equal to or less than pKa_1, when the protonated form is the major species in solution. These results are in good agreement with the numerous references describing the preference of CBs for binding positively charged guests due to charge stabilization by the negative carbonyl-bearing portals of the CB [12,13]. In contrast, slight and almost negligible fluorescence increases were observed at pH values corresponding to the neutral and negatively ionized forms of carbendazim. Of all the pH's assayed, pH 4.0 (0.2 M acetate buffer) was found to produce the strongest analytical signal, therefore this value was taken to be the optimal pH value for CBZ determination. Fig. 3 shows the typical spectra recorded for increasing CB[7] ratios under these latter conditions.

In contrast to CDs, the CBs' charged portals influence their interactions with host molecules to a large degree. The competing ion–dipole interaction between the ionic reaction medium and the analyte to be hosted by CBs has been reported previously [23,24], therefore the influence of the acetate buffer concentration on complex formation was also investigated by recording fluorescence spectra for CBZ-CB[7] solutions with acetate buffer concentrations in the range 0.0–0.5 M. As expected, a low ionic concentration was found to favour complex formation due to the reduction in the competing ion–dipole interactions (Fig. 4), therefore, it was decided to perform the remaining studies with an acetate buffer concentration of 10^{-4} M.

3.2.2. Stoichiometry studies

The fluorescence data measured for increasing CB[7] concentrations allowed stoichiometry studies to be performed. Several

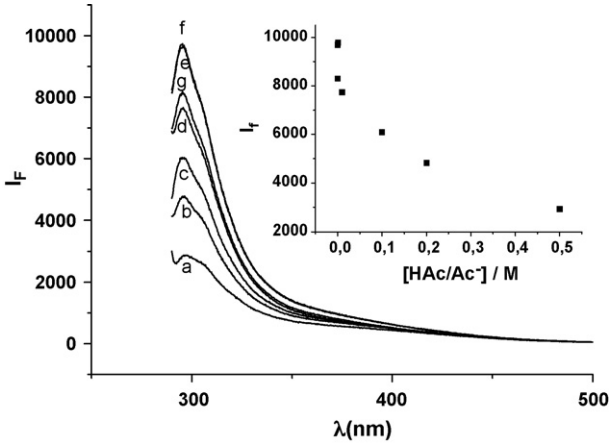

Fig. 4. Influence of acetate buffer concentration on CBZ-CB[7] complex formation and fluorescence intensity. (a) 5×10^{-1} M; (b) 2×10^{-1} M; (c) 1×10^{-1} M; (d) 1×10^{-2} M; (e) 1×10^{-3} M; (f) 1×10^{-4} M; (g) 10^{-4} M HCl.

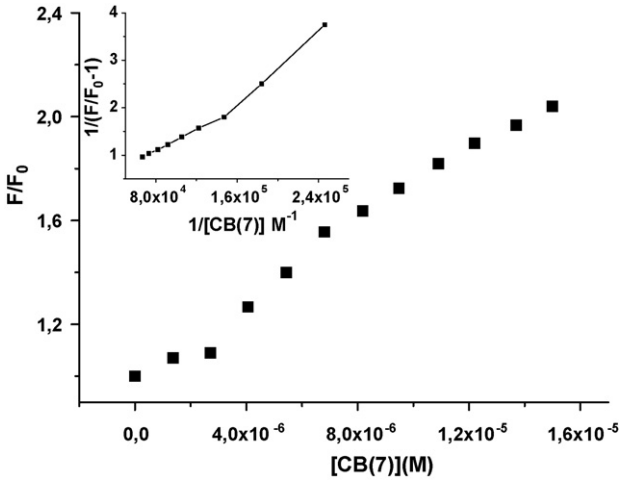

Fig. 5. Fluorescence enhancement, F/F_o, of CBZ with increasing CB[7] concentration and the nonlinear double reciprocal plot indicating the formation of higher-order complexes (inset) [22].

authors have reported the formation of 1:1 and 2:1 host–guest CB[n] complexes based on the shape of the plot of the fluorescence enhancement produced in the presence of different amounts of CB[n] [25,26]. If only 1:1 host–guest complexes are formed, a linear double reciprocal plot of the fluorescence enhancement data should be obtained; if other lineshapes are obtained then higher order complexes are likely to be present in solution. Fig. 5 shows the fluorescence enhancement of CBZ as a function of CB[7] concentration and the nonlinear double reciprocal plot obtained (inset). As mentioned above, these results suggest the formation of 1:1 and 2:1 host–guest complexes. Similar results have been reported previously for inclusion complexes between CBZ and β-CD [27].

3.3. Validation

The analytical performance of the developed method for carbendazim determination was evaluated under the optimized conditions described above, with a 10-fold excess of CB[7] with respect to CBZ in all assays. With this excess, the I_f intensity remains constant at all the initial CBZ concentrations in the linear range which ensures that changes on I_f are due to Lambert–Beer's law. A linear increase of the fluorescence intensity was observed in the concentration range [28] 2.6×10^{-8} to 2.2×10^{-6} M, according to the equation $I_f = 113.3 + 4.3 \times 10^9$ M ($r = 0.9994$), with increasing CBZ-CB[7] concentration. The high sensitivity of the proposed method was inferred from the calculated LOD ($\bar{x}_b + 3\sigma$) and LOQ ($\bar{x}_b + 10\sigma$) values of 5.0×10^{-9} and 2.6×10^{-8} M, respectively. These values were calculated with the standard deviation of the blank signal [29]. The RSD (%) and Er (%) ($n = 5$) values were evaluated at different concentrations (5.2×10^{-8}, 2.6×10^{-7}, 5.2×10^{-7}, 1.1×10^{-6} and 2.1×10^{-6} M; see Table 1), and the calculated values show the high reproducibility of the method irrespective of the concentration assayed. Acceptable Er (%) values were obtained when working with very low concentrations (i.e. 13% at a concentration of 5.2×10^{-8} M). These results show that the proposed method can be applied with sufficient accuracy and reproducibility.

Table 2
Maximum concentration tolerated for other pesticides to produce interference in CBZ determination.

Interference	Maximum allowed concentration (M)
Thiabendazole	5.2×10^{-7}
Thiamethoxam	2.6×10^{-5}
Benomyl	1.3×10^{-7}
Diuron	2.6×10^{-5}

3.3.1. Interference study

The effect of various substances that could be present in the samples and interfere with the carbendazim determination was evaluated. Thus, increasing amounts of other benzimidazole fungicides such as thiabendazole, diuron, thiamethoxam and benomyl were added to a solution containing 5.2×10^{-7} M CBZ-CB[7]. A foreign substance was considered to produce interference when a variation of the complex fluorescence of 10% or more was recorded. The results obtained in these experiments are summarized in Table 2.

In contrast to thiabendazole, which interfere the CBZ determination at a 1:1 concentration ratio, diuron and thiamethoxam do not interfere with CBZ determination (decreasing fluorescence intensity) until they reach very high levels with respect to CBZ. However, the presence of benomyl influences the CBZ determination to a much greater extent, which is not surprising as carbendazim is the main degradation product of benomyl. Moreover, benomyl tolerance is usually expressed in terms of CBZ [17].

4. Analytical application

Matrix solid phase dispersion (MSDP), which combines homogenization, cellular disruption, extraction, fractionation and purification in a single process [30], was selected as the sample-preparation procedure. Several authors have reported MSPD procedures for CBZ determination in different samples such as fruit juices [31], fruits [32] or wheat grains [33] using C8, C18 or acidic silica gel as sorbents. The MSDP procedure developed in this work for CBZ extraction from orange samples was described in Section 2.3.2.

The standard addition method was employed for CBZ determination. Procedural (i.e. reagents) blank and orange blank samples were analysed in each set of experiments to check contamination throughout the analytical method. No background interference was found to be introduced by the methodology proposed. The fluorescence data recorded during analysis of unspiked samples and samples spiked with 0.25, 0.5, 0.74 and 0.99 μg CBZ showed a linear increase in the fluorescence intensity according to $I_f = 362.3 + 669.9$ μg CBZ ($r = 0.998$). The LOD ($\bar{x}_b + 3\sigma$) and LOQ ($\bar{x}_b + 10\sigma$) calculated for the real sample (Table 3) showed that the minimum detectable concentration was 0.10 mg/kg, which is lower than the maximum legal level of 0.5 mg/kg allowed for this kind of sample [34]. Although, somehow low recoveries were found (in the 31–55% range) these results agree with those previously reported in the literature for similar applications [32]. On the other hand, really satisfactory reproducibility (RSD values below 7%) proved that the proposed method can be applied with enough precision to the analysis of real samples at low concentration levels.

Table 1
Analytical data used to calculate the accuracy and precision of the proposed method at different concentration levels (M).

Er (%)					RSD (%) ($n = 5$)				
5.8×10^{-8}	2.6×10^{-7}	5.2×10^{-7}	1.1×10^{-6}	2.1×10^{-6}	5.8×10^{-8}	2.6×10^{-7}	5.2×10^{-7}	1.1×10^{-6}	2.1×10^{-6}
13	9.4	5.8	1.5	11.5	1.8	2.1	1.7	2.7	2.0

Table 3
Analytical data (referred to wet sample) for CBZ determination in orange samples.

Linear range (mg/kg)	LOD (mg/kg)	LOQ (mg/kg)	Recovery (%)[a]	RSD (%) (n = 3)
0.15–0.60	0.10	0.52	31–55	3–7

[a] Minimum and maximum values of the mean obtained from triplicate measurements for each spiked level.

5. Conclusions

Host–guest interactions between CB[7] and CBZ have been employed to develop a sensitive and selective method for CBZ determination in real samples. The protonated form of CBZ leads to the most stable complex, with a high calculated association constant. No inclusion complex is formed with the anionic form. In addition to a 1:1 complex, high CB[7] concentrations in solution lead to a 1:2 coordination stoichiometry. The proposed method, which involves MSPD during sample preparation, can be applied to CBZ determination in orange samples with sufficient reproducibility and sensitivity to meet the legal requirements for such assays.

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