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γ-Cyclodextrin Inclusion of Phloroglucinol: Solid State Studies and Antioxidant Activity throughout the Digestive Tract

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Abstract: Phloroglucinol is a powerful antioxidant compound and an active pharmaceutical ingredient used in the management of intestinal spasms. In this report, we describe the interaction of γ-cyclodextrin with phloroglucinol to readily form a solid inclusion compound with 1:1 by co-dissolution and freeze-drying. Solid-state characterisation using FT-IR, thermal analyses (TGA and DTA) and X-ray powder diffraction confirmed the formation of a true inclusion compound (γ-CD·PG) in which the molecules of γ-CD are stacked into channels. This spatial arrangement is typical of γ-CD inclusion compounds, and it allows for the guest molecules to be located inside these channels. The evaluation of the antiradical potential of γ-CD·PG (against $O_2^{\bullet}-$ and $NO^{\bullet}$) on different steps of the digestive process (mouth, gastric and intestinal phases) led us to conclude that the inclusion of phloroglucinol promoted better antioxidant activity at the end of the digestion when compared to the free phloroglucinol.

Keywords: cyclodextrin inclusion; solid state; polyphenol; antioxidant; gastrointestinal tract; phloroglucinol

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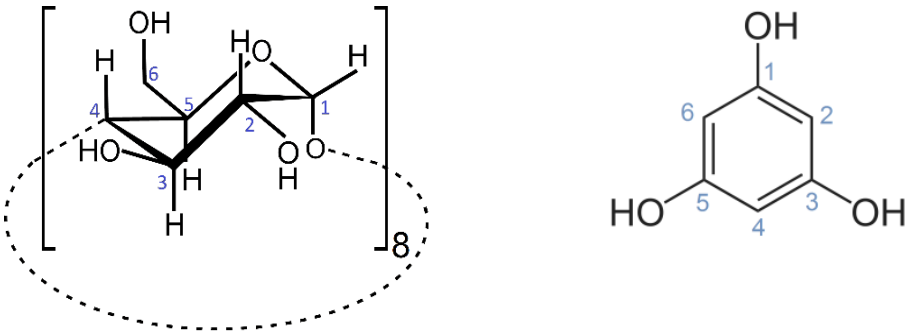
1. Introduction

Phloroglucinol (PG) or 1,3,5-trihydroxybenzene is a natural phenolic compound found in certain plant species, bacteria and brown algae [1] and is used as an antispasmodic agent in commercial pharmaceutical formulations. It is available as a non-prescription medicine n the form of 80 mg tablets with therapeutic indication for scenarios of contractions of the intestine, bile ducts, urinary tracts and uterus [2].

The efficacy of phloroglucinol is particularly relevant in patients with irritable bowel syndrome (IBS), namely regarding its ability to reduce pain [3,4], bloating and mucus production [3] as well as its regulating action on the passing of stool [3]. The antispasmodic action of phloroglucinol can also be useful in the prevention of miscarriages in women with the threatened abortion condition (typically older women are at higher risk): phloroglucinol acts by inhibiting uterine contractions and is safer and more effective than other drugs, such as magnesium sulphate [5].

The phenolic nature of phloroglucinol makes it a powerful antioxidant. In vitro studies showed a ferric reducing ability of plasma value of 96 μM eq. $FeSO_4$ as early as within 4 min of the assay, rising to 365 μM eq. $FeSO_4$ after 30 min, and a radical scavenging ability, at a concentration of 250 μM, of 443 μM Trolox equivalents in the Trolox equivalent antioxidant capacity assay [6]. Moreover, this compound was demonstrated to effectively scavenge reactive oxygen species (ROS) in skin cells, protecting them from the damaging effect of UVB exposure [7]. In vivo studies involving the direct administration of phloroglucinol to rat brain showed that it protects midbrain dopaminergic neurons from the neurotoxin 6-hydroxydopamine through direct ROS quenching and by up-regulating the expression of the antioxidant enzymes catalase and glutathione, thus, hinting at a possible anti-Parkinson effect [8].

Encapsulation of phloroglucinol can offer protection against premature oxidation and extend it for controlled release. Thus far, phloroglucinol encapsulation is reported only by cross-linking with starch with a strong reduction in the antioxidant activity because the hydroxyl groups involved in the cross-linking bonds becomes unavailable for radical scavenging [9]. In this work, we present an encapsulation strategy based solely on non-covalent bond interactions: the formation of supramolecular adducts with cyclodextrins. Formed by bacterial or enzymatic degradation of starch, cyclodextrins are natural cyclic oligosaccharides, typically occurring with six (α-CD), seven (β-CD) or eight (γ-CD, Figure 1a) D-glucose units linked together by α-1,4 glycosidic bonds. They present the shape of a truncated cone, with the secondary hydroxyl groups facing the wider rim, the primary hydroxyls at the narrower rim and the cavity lined with protons. Cyclodextrins are, thus, water soluble, and they can help solubilise other molecules that have an adequate size to fit in their cavity [10,11]. Cyclodextrins can be further used as stabilising and taste-masking agents for a variety of drugs and cryopreservatives or co-adjuvants in vaccines [12–17]. The properties of cyclodextrins make them also very useful in the formulation of food products, particularly those that have been fortified in order to have nutraceutical activity [18,19]. Cyclodextrins have been intensively employed in helping formulate plant active compounds, such as flavonoids [20–22], carotenoids [23,24], terpenoids [25] and a vast range of polyphenols [26–29]. In food applications, the use of γ-CD is preferred over the other native cyclodextrins owing to its higher digestibility and the fact that it can be ingested without any dose limits.



(**a**) (**b**)

Figure 1. Chemical structure and atom numbering of (**a**) γ-cyclodextrin and (**b**) phloroglucinol.

The formation of the inclusion compound γ-CD·PG and its effect on the radical scavenging activity of the guest, phloroglucinol, is herein presented. Structural characterisation of this host–guest system was conducted both in solution and in the solid state. The radical scavenging activity was studied in different media. The media followed a sequence that simulates the gastro-intestinal tract because the inclusion compound is aimed at a future application in food fortification. The results show that the inclusion compound is readily formed and that it lowers the radical scavenging action of phloroglucinol, hinting at a sustained release effect.

2. Materials and Methods

2.1. Materials

Pharmaceutical-grade γ-CD (Cavamax W8 Pharma) from Wacker-Chemie was kindly donated by Ashland Specialty Ingredients (Düsseldorf, Germany). Phloroglucinol was obtained from sigma (St. Louis, MO, USA). Ultrapure water was used for the inclusion procedures. All organic solvents were of analytical grade, except otherwise specified.

2.2. Equipment

Laboratory powder XRD data were collected at ambient temperature on an Empyrean PANalytical diffractometer (Cu $K\alpha_{1,2}$ X-radiation, λ_1 = 1.540598 Å; λ_2 = 1.544426 Å) equipped with an PIXcel 1D detector and with the sealed tube operating at 45 kV and 40 mA (Bruker AXS, Karlsruhe, Germany). Intensity data were collected by the step-counting method (step 0.01°), in continuous mode in the ca. $3.5 \leq 2\theta \leq 50°$ range.

Infrared spectra were obtained as KBr pellets in a 7000 FTIR spectrometer (Mattson, Oakland, CA, USA) (resolution 2.0 cm^{-1}; 128 scans per spectrum).TGA studies were performed on a Hitachi STA-300 analyser (Tokyo, Japan), using a heating rate of 5 C min^{-1}, under air atmosphere. Data from the spectrophotometric experiments was acquired in a Biotek, automatic microplate reader (Vienna, Austria).

Two-dimensional NMR Nuclear Overhauser Effect Spectroscopy (NOESY) data was recorded on a Bruker Avance 500 spectrometer at 500.13 MHz at room temperature on a solution with equimolar quantities of phloroglucinol and γ-CD (concentration 13.6 μM, solvent D_2O).

2.3. Preparation of the Inclusion Complex of γ-CD with Phloroglucinol as a Solid Material

A solution of γ-CD (715.1 mg, 0.5 mmol) in ultrapure water (10 mL) at 40 °C was treated with phloroglucinol (63.6 mg, 0.5 mmol). The mixed solution was stirred for 3 min and then subjected to snap-freezing in liquid nitrogen. The frozen water was subsequently removed by freeze-drying to obtain a white solid.

2.4. Gastrointestinal Digestion Simulation

Simulation of gastrointestinal digestive system was conducted following an adaptation of the study of Catarino et al. [30]. The process started with the oral digestion by suspending 0.5 g of dried sample (PG or γ-CD.PG) in 10 mL of distilled water, followed by a pH adjustment to 5.6–6.9 with 1M $NaHCO_3$, and the addition of 0.3 mL/min of α-amylase at 100 U/mL. This digestive step was carried out for 2 min at 37 °C and with constant agitation at 200 rpm. Before moving to the gastric compartment, the mouth digest pH was adjusted to 2.0 with 1M HCl and mixed with a simulated gastric juice consisting of pepsin 25 mg/mL added at a ratio of 0.05 mL/mL of mouth digest. This mixture was then incubated for 60 min at 37 °C under agitation at 130 rpm.

Finally, for the intestinal digestion, another pH adjustment to 6.0 was necessary using 1M $NaHCO_3$ prior to the addition of a simulated intestinal juice consisting of 2 g/L of pancreatin and 12 g/L bile salts at a ratio of 0.25 mL/mL of gastric digest. The samples were then incubated during 120 min, at 37 °C and 45 rpm, to mimic a long intestine digestion process. An aliquot of 2 mL was collected after each step of digestion, i.e., mouth digest, gastric digest, intestinal digest. All samples were freeze dried and stored in a desiccator until further use.

2.5. Antioxidant Assays

The $NO^{\bullet}$ scavenging method was adapted from Catarino et al. [31]. For this, 100 μL of six different sample concentrations (0–1 mg/mL) was mixed with 100 μL of sodium nitroprusside (3.33 mM in 100 mM sodium phosphate buffer pH 7.4) and incubated for 15 min under a fluorescent lamp (Tryun 26 W). Next, 100 μL of Griess reagent (0.5% sulfanilamide and 0.05% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4) was added to the mixture, which was incubated for another 10 min at RT in the dark. The absorbance was then measured at 562 nm, and the $NO^{\bullet}$ scavenging capacity was calculated as the concentration of the sample capable of scavenging 50% of the radical.

The $O_2^{\bullet -}$ scavenging method was carried out according to the method described by Pereira et al. [32]. In a 96-well plate, 75 μL of six different sample concentrations (0.0–2.0 mg/mL) was mixed with 100 μL of β-NADH (300 μM), 75 μL of NBT (200 μM) and 50 μL of PMS (15 μM). After 5 min, the absorbances at 560 nm were recorded and the inhibition calculated as the concentration capable of scavenging 50% of $O_2^{\bullet -}$ (IC_{50}).

2.6. Statistical Analysis

Data was expressed as mean ± standard deviation (SD) of three similar and independent experiments using a student *t*-test one-way for the experiments with the non-digested samples and one ANOVA followed by Tukey's post hoc test for the experiments with digested samples. The statistical tests were applied using GraphPad Prism, version 6.01 (GraphPad Software, San Diego, CA, USA), and the significance level was $p < 0.05$.

3. Results and Discussion

3.1. Investigation of Phloroglucinol Inclusion into γ-CD in Aqueous Solution

The interaction of phloroglucinol with γ-CD was first investigated in aqueous solution. For this, we resorted to NOESY (Nuclear Overhauser Effect Spectroscopy), a 2D NMR technique that allows observing molecular interactions through space. The spectrum for a D_2O equimolar solution of phloroglucinol and γ-CD mixture, depicted in Figure 2, shows evidence of inclusion. The H5 and H6 protons of γ-CD appear as a single resonance with a maximum at 3.75 ppm, slightly shifted in comparison with the values reported for pure γ-CD (3.56–3.65 ppm) [33,34]. Note that the H5 protons are located inside the cavity of γ-CD, and therefore a shift in their signal is associated with the formation of inclusion complexes in solution. The shift in H6 protons suggests that phloroglucinol may be interacting with the primary hydroxyl side of γ-CD [34]. Furthermore, NOESY correlations were found between the H5 protons of the host and the benzene protons of phloroglucinol, which corroborates inclusion of the benzene ring into the cavity of γ-CD.

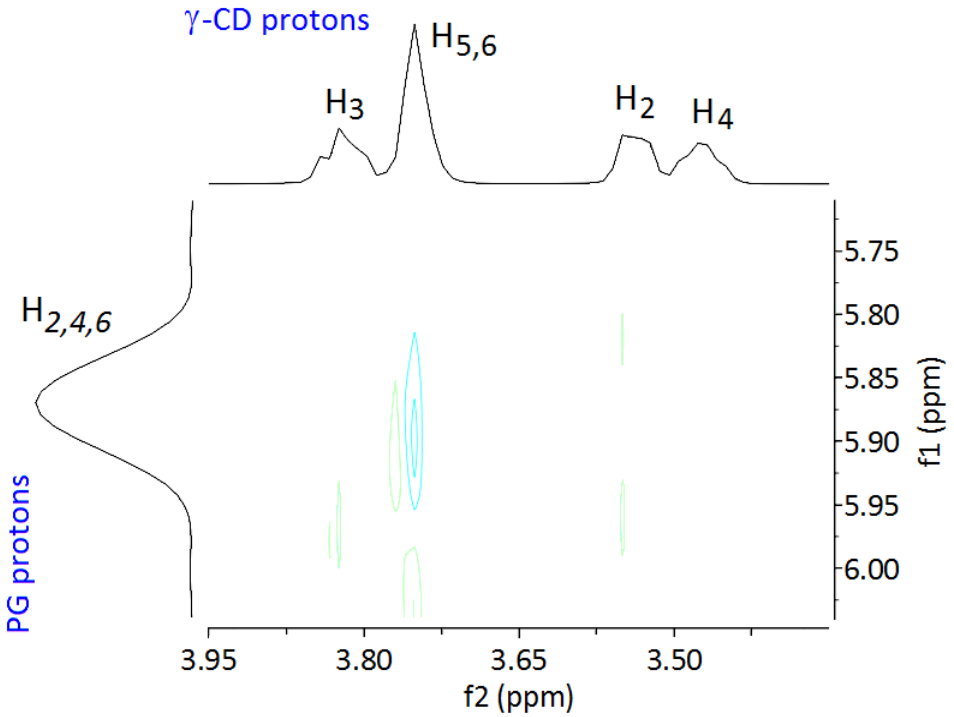


Figure 2. NOESY spectrum of a D_2O solution containing phloroglucinol (PG) and γ-CD (both at a concentration of 13.6 mM).

3.2. Preparation of the γ-CD·Phloroglucinol Inclusion Compound as a Solid

The information gathered from solution studies indicated that, in aqueous solution, γ-CD and phloroglucinol interact readily to form an inclusion complex. Thus, preparation of the corresponding solid was done by a simple process of co-dissolution in aqueous solution at 40 °C. Upon freeze-drying the mixed solution, a bulky white solid was obtained that corresponded to the inclusion compound of these two components, as verified by powder XRD, FTIR and thermal analyses. The product is hereafter denominated as γ-CD·PG.

3.3. Solid-State Studies of γ-CD·Phloroglucinol Inclusion Compound

3.3.1. FT-IR Spectroscopy

Infrared spectroscopy is a useful tool for quick investigation regarding inclusion compound formation. Guests with oscillators that (i) are sensitive to the hydrophobic environment of the host cavity and (ii) present bands located in a spectral area devoiced of any overlapping host bands are particularly suited to be analysed under this spectroscopic method.

A comparison of the spectra of PG and γ-CD·PG permits noting that two bands, assigned to aromatic νCC and observed at 1498 and 1537 cm^{-1} in pure phloroglucinol [35], suffer redshifts of 5 and 7 cm^{-1}, respectively, upon formation of the inclusion compound with γ-CD (Figure 3) [36–39]. The lower vibrational energy of these oscillators in γ-CD·PG indicates the breaking of the inter-molecular hydrogen bonds (that occurred in pure phloroglucinol [35]) due to the encapsulation of each individual molecule of this guest into the cavity of a cyclodextrin molecule [36–39].

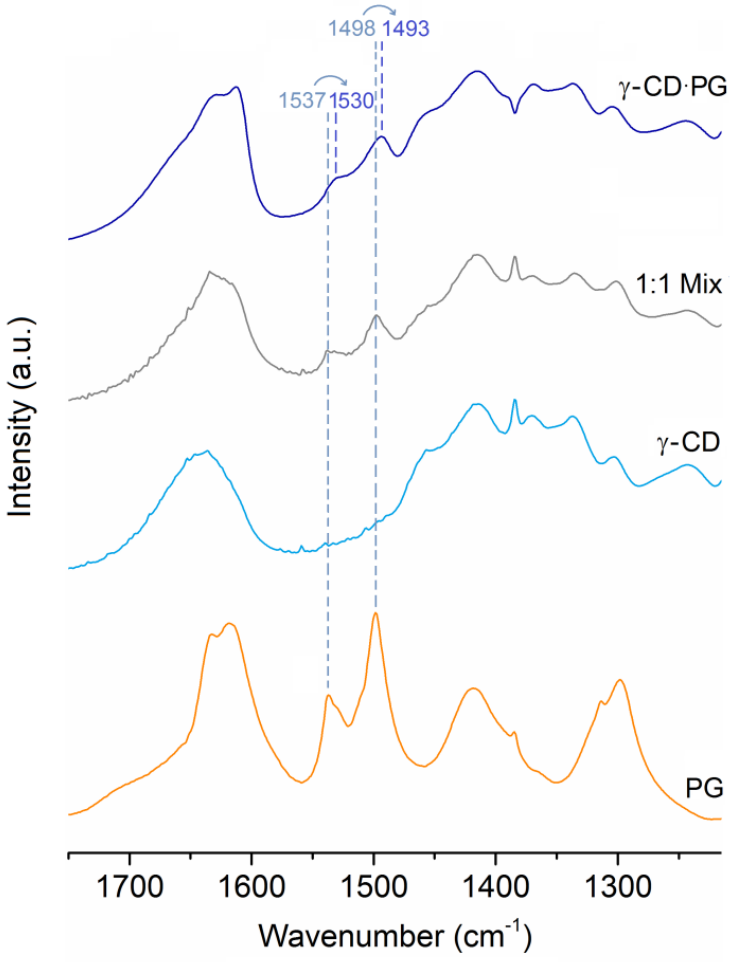


Figure 3. Fourier-transform infrared spectra of phloroglucinol (PG), γ-CD, their 1:1 physical mixture (1:1 mix) and the freeze-dried solid products starting from mixed solutions with a γ-CD:PG ratio of 1:1 (γ-CD·PG).

3.3.2. Powder X-ray Diffraction

Powder X-ray diffraction (PXRD) is a useful tool in identifying the formation of inclusion complexes. It also contributes to investigating the presence of any eventual impurities that may appear, such as crystallites of the host or the guest. PXRD data is presented in Figure 4.

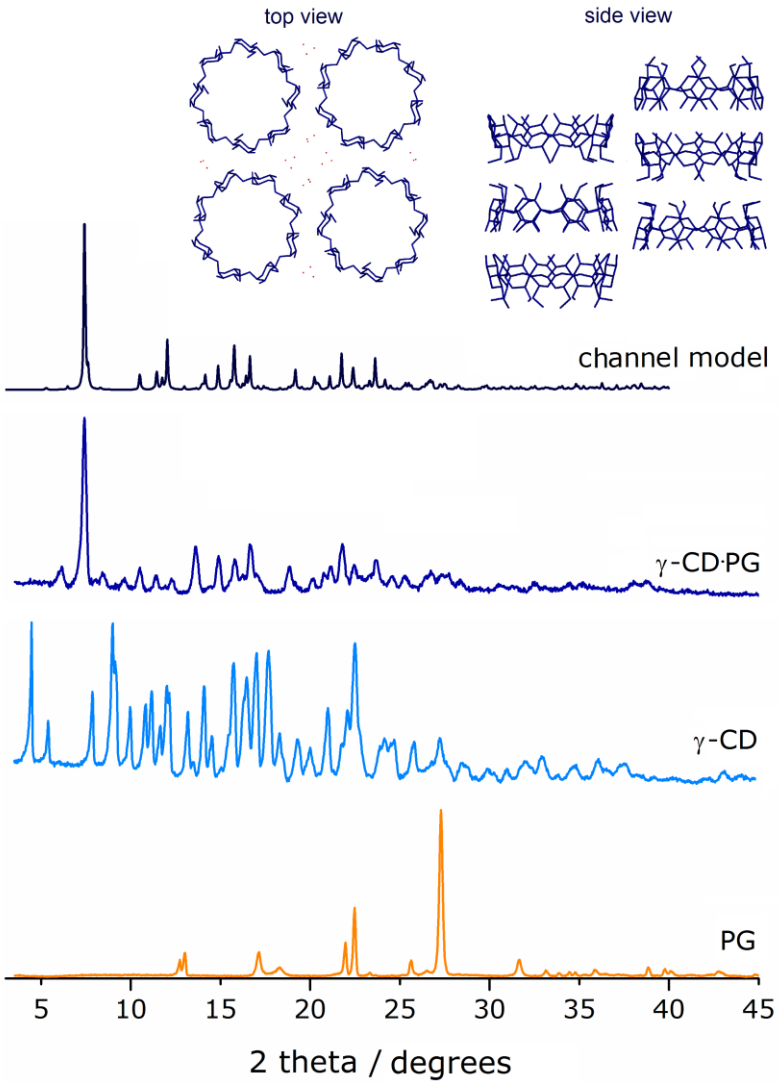


Figure 4. Experimental powder X-ray diffractograms of phloroglucinol (PG), γ-CD and the inclusion compound γ-CD·PG. The diffractogram on top of the image is the calculated trace of the inclusion complex γ-CD·12-crown-4-ether [40] starting from its atomic coordinates (data available at the CCDC under the refcode DOCYD) and using the software Mercury 3.5.1 (Copyright CCDC 2001–2014). The inset depicts the spatial arrangement of cyclodextrin molecules in the crystals of compound γ-CD·12-crown-4-ether, as viewed from the top (crystallographic *c* axis) and from the side (*a* axis).

Phloroglucinol presents a diffractogram with several well-resolved peaks that are indicative of its high crystallinity. The most intense reflections occur at 12.7, 13.0, 17.2, 18.3, 22.0, 22.5, 25.7, 27.2 and 31.7 degrees of 2θ.

The diffractogram of the product of γ-CD·PG, as obtained after the freeze-drying process, revealed a mainly amorphous phase (which was expected as a result of the preparation method) that was lacking reflections that would allow for an adequate structural analysis. To restore the hydration waters and some of the crystallinity of this sample, it was placed at ambient temperature in a water-saturated atmosphere for approximately 16 h. The PXRD pattern of the rehydrated γ-CD·PG showed a set of diffraction peaks corresponding to the formation of a new phase with no traces of crystallites of γ-CD heptahydrate. The main reflections presented maxima at 6.2, 7.5, 8.5, 9.6, 10.5, 11.4, 12.3, 13.7, 14.9, 15.8, 16.6, 18.8, 20.2, 20.7, 21.2, 21.8, 22.4 and 23.6 degrees of 2θ. The overall diffraction envelope for this sample had a fair degree of similarity with the one calculated for γ-CD·12-crown-4-ether [40], herein used as a representative model for the only known isostructural series of γ-CD inclusion complexes [41]. This similarity suggests that γ-CD·PG belongs to this

isostructural series; thus, we assume that it has γ-CD molecules stacked in infinite channels as reported for all the inclusion compounds of this isostructural series (inset in Figure 4).

3.3.3. Thermal Analyses

The thermograms of phloroglucinol, γ-CD, their 1:1 physical mixture and the inclusion compound γ-CD·PG are represented in Figure 5. The thermogram of pure phloroglucinol shows an initial dehydration step of 15% and then no further mass loss until about 200 °C, the temperature that marks the onset of its decomposition. The decomposition step of phloroglucinol compound is absent in the thermograms of both the inclusion compound, γ-CD·PG, and the physical mixture.

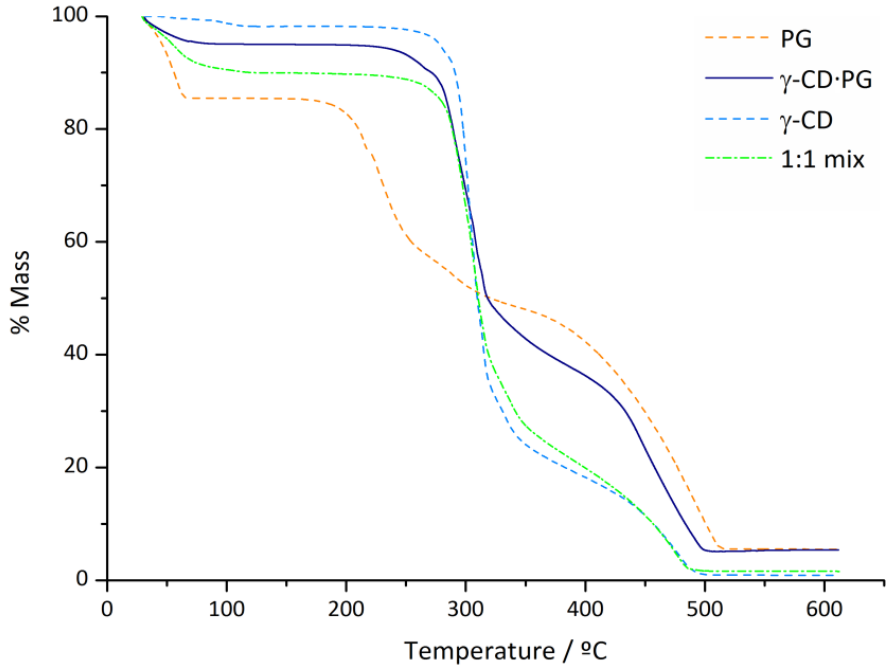


Figure 5. Thermogravimetric traces of phloroglucinol (PG), γ-CD, their 1:1 physical mixture (1:1 mix) and inclusion compound γ-CD·PG.

While for γ-CD·PG the absence of the guest decomposition step is expected because of the inclusion of the guest molecules in the channels of γ-CD, with subsequent slow or no release until the decomposition of the structure of the host, the feature is far less common in a physical mixture. This may be indicative of a strong affinity of this guest to the host, γ-CD, with possible in situ formation of an adduct over the course of thermogravimetric data acquisition.

Phloroglucinol, γ-CD, their 1:1 physical mixture and the inclusion compound γ-CD·PG were further studied using DTA. The results are represented in Figure 6.

Pure phloroglucinol shows two endothermal peaks. The first one, centred at 60 °C, corresponds to the loss of hydration water molecules, and the second peak occurring at 217 °C, corresponds to its melting transition. The melting peak of phloroglucinol is absent from γ-CD·PG, thus, confirming inclusion; it was also not observed in the trace of the physical mixture, similarly to the results obtained with TGA. This observation corroborates the TGA results and further confirms a strong host-to-guest affinity.

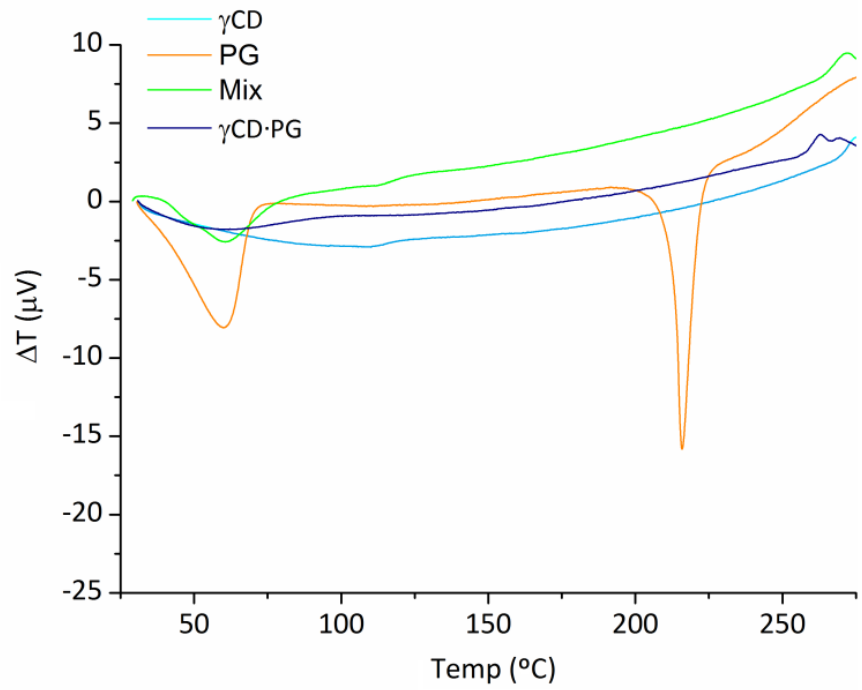


Figure 6. DTA traces of phloroglucinol (PG), γ-CD, their 1:1 physical mixture (1:1 mix) and inclusion compound γ-CD·PG.

3.4. Anti-Radical Activity of Free and Included Phloroglucinol along the Gastrointestinal Tract

The antioxidant activity of a compound, expressed as its ability to interact and neutralise free radicals, can be affected by a broad range of factors. When considering oral intake, simulating the gastrointestinal conditions helps understand how factors, such as the pH of the different digestive fluids and interactions with digestive enzymes, may influence the antioxidant activity. In the case of an inclusion compound, the activity of an antioxidant guest molecule is further modulated by its affinity to the cyclodextrin host: guests with high affinity to their host are released more slowly to the medium, in what is deemed as a sustained release system.

In this section, the effect of gastrointestinal fluids on the ability of phloroglucinol and its inclusion compound, γ-CD·PG, to scavenge two radical species, $O_2^{\bullet -}$ and $NO^{\bullet}$, was studied. For that, the compounds were immersed in a sequence of different media and times of exposure that mimic the digestive path: 2 min in an α-amylase solution to simulate the oral cavity, followed by 1 h in presence of pepsin, to simulate the stomach, and finally the mixture was added with pancreatin and bile salts for 3 h to simulate the intestine.

As expected, the scavenging activity of PG on both $O_2^{\bullet -}$ and $NO^{\bullet}$ scavenging assays decreased throughout the simulated digestive process, as perceived by the increase in the IC_{50} values (Figure 7). This observation agrees with our previous studies on phlorotannins extracted from *Fucus vesiculosus*, which were found to be susceptible to the extreme conditions of the gastrointestinal fluids, consequently losing their antioxidant activity throughout the different compartments of the digestive tract [30]. In fact, the loss of integrity and bioactivity, such as antioxidant, is extremely common among phenolic compounds in general, which are well-known to be degraded and metabolised during their passage through the gastrointestinal tract [42,43].

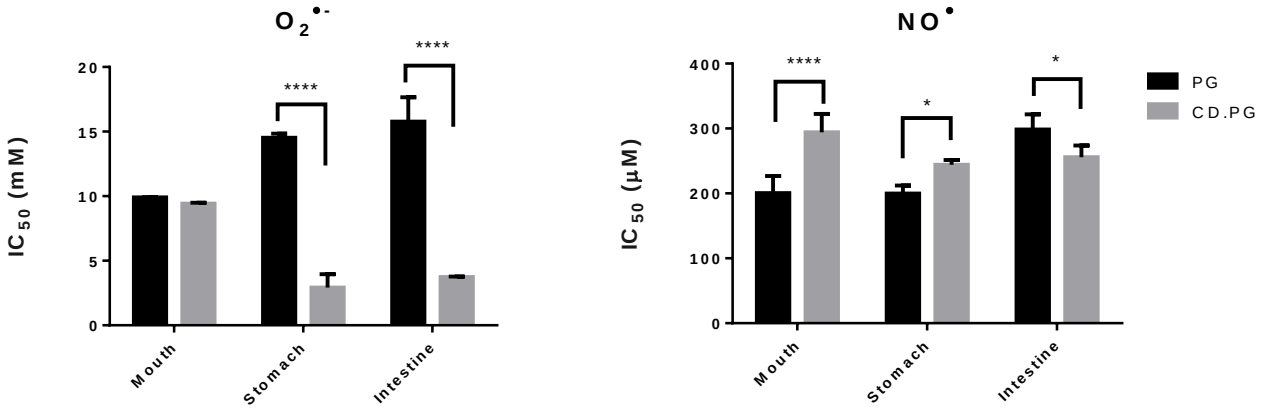


Figure 7. $O_2^{\bullet-}$ and NO• scavenging activities of phloroglucinol (PG) and the inclusion compound γ-CD·PG along the different stages of gastrointestinal digestion. Data represent the mean ± SD of at least three independent assays. * $p < 0.05$ and **** $p < 0.0001$ indicate statistically significant differences.

Molecular encapsulation of PG with γ-CD appears as a possible approach to prevent this compound from being exposed to the gastrointestinal conditions, protecting it from degradation and ensuring its sustained release in the intestinal lumen, where it will be available for absorption. In fact, in both assays, the inclusion compound was shown to exert increased scavenging activity as it progressed from the oral to the gastric conditions, maintaining stable IC_{50} values throughout the rest of the digestive process. These observations could be explained by the sustained-release effect of the γ-CD since exposure to the oral cavity fluid lasts only 2 min, which is a rather low contact time comparing with that from the following compartments (at least 1 h). It also indicates that time is a key factor for the encapsulated phloroglucinol to produce desirable effects.

Interestingly, inclusion of PG into γ-CD resulted in a strong improvement of its scavenging activity against $O_2^{\bullet}-$. This may be attributed not only to the protective effect of γ-CD against PG degradation, but also to a promoting effect of γ-CD on this reaction, in what is attributed to an enzyme mimicry activity of cyclodextrins [44,45]. This effect is not, however, universal. Indeed, this did not occur with the scavenging activity of NO•. This can possibly be explained by the fact that the scavenging activity of PG itself against NO• is far more effective (in the order of μM) than against $O_2^{\bullet}-$ (in the order of mM).

In fact, according to our previous studies on phlorotannins [46], these compounds are capable of inhibiting the radical activity of NO• as effectively as ascorbic acid, which is commonly used as reference compound in this assay. In this case, encapsulation of PG into γ-CD may be contributing to reduce the accessibility of the compound to the NO• substrate, which could explain the lowering of the measured activity during the oral and stomach phases, compared to the free PG. Nevertheless, when it reaches to the intestinal phase, γ-CD·PG was able to maintain a steady NO• scavenging activity, while free PG was found to lose its activity, due to the cumulative degradation suffered over the different stages of gastrointestinal digestion.

4. Conclusions

In the present report, we investigated the formation of the γ-CD·PG both in solution and in the solid state. Solution studies were conducted by NOESY on an equimolar aqueous mixture of the two components. The results demonstrated that (i) phloroglucinol is readily included into γ-CD to form an inclusion complex and (ii) inclusion involves interaction of the benzenic ring of phloroglucinol with the H5 protons of γ-CD. The results from NOESY demonstrated that γ-CD·PG can be obtained by a process of co-dissolution in water, with no need for any additional co-solvent. Subsequent snap-freezing and freeze-drying to remove the water solvent allowed isolating the inclusion compound as a solid product. Powder

X-ray diffraction further showed that the compound γ-CD·PG belongs to the isostructural series of γ-CD inclusion compounds with tetragonal symmetry, in which γ-CD molecules form channels that hold the guest molecules inside. This contributed to the redshift of two vibrational bands of phloroglucinol as observed by FT-IR.

When submitted to simulated digestive fluids, it became clear that encapsulation with γ-CD protects PG from the extreme conditions of the gastrointestinal tract allowing a sustained-release of the compound along the gastrointestinal tract, resulting in a better antioxidant activity towards $O_2^{\bullet}-$ and $NO^{\bullet}$ at the end stage—the intestinal fluid. The controlled release of PG from the γ-CD appears to be mainly affected by contact time. Overall, this study indicated that encapsulation of PG with γ-CD could be a viable approach to improve the digestive stability and intestinal transport of phloroglucinol, preserving its bioactive properties and particularly its antioxidant activity.

Author Contributions: Conceptualisation, A.R.C., S.S.B., S.M.C.; methodology, B.S.B.C., A.R.C., M.D.C.; data curation, B.S.B.C., M.D.C., S.S.B.; formal analysis, B.S.B.C., M.D.C., S.S.B., S.M.C.; investigation, B.S.B.C., A.R.C., M.D.C.; writing—original draft preparation, S.S.B., M.D.C.; writing—review and editing, M.D.C., S.S.B., S.M.C., A.M.S.S.; supervision, M.D.C., S.S.B., S.M.C.; funding acquisition, S.M.C., A.M.S.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Solid-state characterisation data are available upon request from the PXRD and thermal analyses services of the University of Aveiro.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

DTA	Differential thermal analysis
FT-IR	Fourier-transform infrared spectroscopy
γ-CD	gamma-Cyclodextrin
γ-CD·PG	Inclusion compound of phloroglucinol in gamma-Cyclodextrin
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
PG	Phloroglucinol
PXRD	Powder X-ray diffraction
TGA	Thermogravimetric analysis

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