Communication

Diester Chlorogenoborate Complex: A New Naturally Occurring Boron-Containing Compound

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Abstract: The natural compounds of boron have many applications, including as dietary supplement. Communication is based on the discovery that diester chlorogenoborate complex can be detected and quantified from green coffee bean. The study reports that such diester molecule can also be synthesized in stable form by direct reaction of boric acid and chlorogenic acid, in a mixture of acetonitrile–water (1:1, *v*/*v*), left to evaporate over a period of 48 hours, at room temperature, resulting a spirocyclic form (diester complex). The diester complex, with its molecular structure and digestibility attributes has potential application as prebiotic in gut health, oral health and as micronutrient essential for microbiota to human and animals.

Keywords: diester chlorogenoborate complex; green coffee bean; HPTLC/UV; HPTLC/ESI–MS; UHPLC/MS; FTIR

1. Introduction

Boron (B) organic species are present in plants across a large range of essential primary metabolites including B–carbohydrate complexes [1, 2] and B amino acids [3, 4], as well as secondary metabolites, such as organic acids [5, 6] and still undiscovered *in vivo* as B phenolic compounds [7, 8].

Phenolic acids are found in plants and exert a significant biological function in achieving communication between plants and other organisms [9, 10]. At the same time,

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phenolic acids act on microbiota through the suppression of predators and pathogens, as well as the stimulation of mycorrhizae [11–13].

Chlorogenic acid (5-O-caffeoylquinic acid, CA) is a simple phenolic compound included in the hydroxycinnamic acid family and demonstrates biological activity beneficial for human health, such as anti-inflammatory, antioxidant, antidiabetic, antilipidemic, antimicrobial and antihypertensive [14].

The metabolism of CA has been well explored, and through these studies shown to be metabolized mainly by the colonic microflora [15]. Furthermore, CA has been shown to be a major prebiotic involved in the rise of *Clostridium coccoides–Eubacterium rectale*, and *Bifidobacterium* spp. [16].

The high B complexation capacity of CA and the use of CA in the analytical chemistry of B determination [17, 18] have led us to search in plants with high concentrations of B and CA, as it is well known that phenol metabolism needs B to for a healthy plant metabolism. It is also known that the lack of B in the metabolism of phenols in the plant causes their oxidation. At the same time, the formation of B complexes determines phenolics' unavailability to oxidation [19, 20].

In the literature, it has been established that: (*i*) the coupling of phenolic acids (3,4-dihydroxybenzoic acid, caffeic acid and CAs) and boric acid (BA), when observed by spectroscopy, yields a 1:1 assembly in every reaction [7]; (*ii*) with the first two acids (3,4-dihydroxybenzoic acid and caffeic acid), there is evidence that a 2:1 compound also resulted at significant acid-to-B proportion, however for CA no 2:1 diester complexes were identified [7]; (*iii*) catechol derivatives, CA and caffeic acid only form mono-borate compounds [21]. Subsequently, existing studies do not signal the *in vitro* formation of CA diester with B. Previous work did not find evidence for the formation of 1:2 BA complexes with catechol's (B phenolic diesters) and substituted catechol's that had been examined.

Our communication is based on the discovery and semisynthesis of a new natural B phenolic compound – diester chlorogenoborate (DCB) complex – which, being a complex of B with a phenolic acid, can be nutritive prebiotic for human and animal microbiota [22]. We chose only plants with a B concentration higher than 10 ppm and high phenolic acids to increase the possibility of identifying new B compounds (especially B-containing phenolic acid compounds). After the rigorous tests that were highlighted in a previous report, we chose only the green coffee bean (GCB) as it contained the highest amount of B organic species and CAs [23].

2. Aim

We present here a method for DCB semisynthesis and rapid identification from GCB by using the high-performance thin-layer chromatography (HPTLC)/ultraviolet (UV) densitometry with confirmation by online HPTLC/electrospray ionization (ESI)–mass spectrometry (MS) and ultra-high-performance liquid chromatography (UHPLC)/MS technique.

3. Materials and Methods

3.1. Chemicals and solvents

CA standard (98% purity) was purchased from Alfa Aesar (Thermo Fisher GmbH, Kandel, Germany). BA standard and solvents (2-propanol, acetonitrile, water – LiChrosolv® grade; formic acid – EMSURE® grade) were all purchased from Merck (Burlington, Massachusetts, USA). The HPTLC Si 60 F_{254} 20×10 cm glass plates were also obtained from Merck.

3.2. Plant material and GCB extract

The product in which we identified and quantified DCB was the Ethiopian Djimmah GCB purchased from an online market. The GCB extract was prepared by macerating 10 g of powdered GCB, overnight, with 90% acetonitrile.

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3.3. Semisynthesis of DCB

CA and BA were solubilized in a mixture of acetonitrile–water (1:1, *v*/*v*). The mixture was then left to evaporate over a period of 48 hours, at room temperature.

3.4. Methods of analysis

3.4.1. HPTLC/UV densitometry with confirmation by online HPTLC/ESI–MS

The HPTLC/UV densitometry with confirmation by online HPTLC/ESI–MS was performed using a CAMAG (Muttenz, Switzerland) system. Band sample application was accomplished using the CAMAG Linomat 5. Elution was conducted in a 20×20 cm twin-trough chamber with a mixture of 2-propanol–water (8:2, *v*/*v*) with 0.1% formic acid. After the elution, the plate was dried and scanned with the CAMAG TLC Scanner 3 to obtain the densitograms. All the equipment was controlled using the CAMAG VisionCats software package. To confirm the compounds, we used the TLC–MS Interface 2 to directly collect the bands from the plate and introduce them into the mass detector. We tried various mobile phases, the best yielding result was acetonitrile–water (9:1, *v*/*v*) with 0.1% formic acid.

3.4.2. UHPLC/MS analysis

The UHPLC/MS analysis was performed on the Waters (Milford, Massachusetts, USA) Arc System coupled with a Waters QDa with ESI probe. The column was a Waters Cortecs C18 (4.6×50 mm, 2.7 µm) eluting with two solvents: A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient used was: 0–10 minutes 10% to 50% B, 10–11 minutes 50% to 10% B. The mobile phase had a flow rate set at 0.6 mL/min. The column temperature was equilibrated to 20°C. The injection volume was 5 µL. The QDa was set to negative mode at 0.8 kV for the capillary, 50 V for the cone voltage and 400°C for the capillary. The mass range was set at *m*/*z* 100–800.

3.4.3. Fourier-transform infrared (FTIR) spectroscopy

A Frontier Two FTIR spectrophotometer, monitored by a distinctive software, Spectrum v. 10.5.1 (Perkin Elmer, Inc., Boston, Massachusetts, USA), was utilized for analysis. The software permits both quantitative (Spectrum Quant) and qualitative (Spectrum Search) analysis, by detecting the specific bands in comparison with equivalent complexes in the database. The quantitative analysis uses for determination Lambert–Beer law at a certain wavelength or chemometric methods [Principal Component Regression (PCR), Partial Least Squares (PLS) or PLS+], in which the entire obtained spectral range can be used. The samples were examined using the Attenuated Total Reflection (ATR) method, with ZnSe crystal, which permit the IR spectrum recording in the range of 4000–650 cm^{-1} or KRS-5 method, which permit the IR spectrum recording in the range of 4000–400 cm^{-1}. In the above-mentioned IR ranges, 16 scans were performed, with resolution of 4 cm^{-1}.

3.5. In vitro simulation of DCB digestion

In vitro digestion of DCB was performed using a human gastric simulator for stomach (gastric phase) and duodenum (small intestinal phase), according to our previously published paper [22].

4. Results

Figure 1 highlighted the HPTLC/UV densitometry analysis of BA, CA, DCB, GCB extract and of GCB extract with BA.

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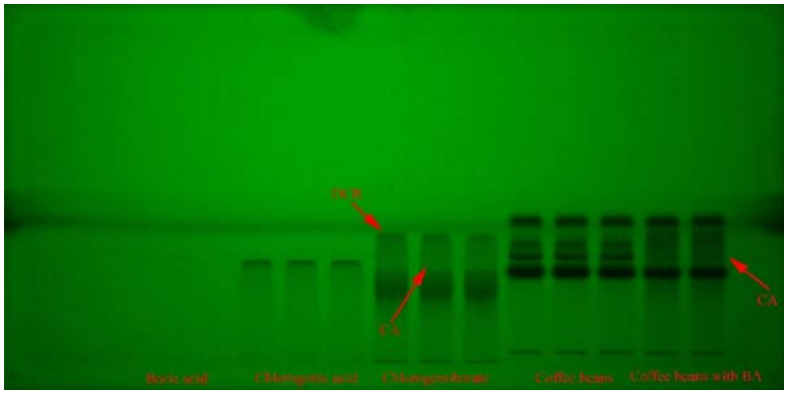
(a)



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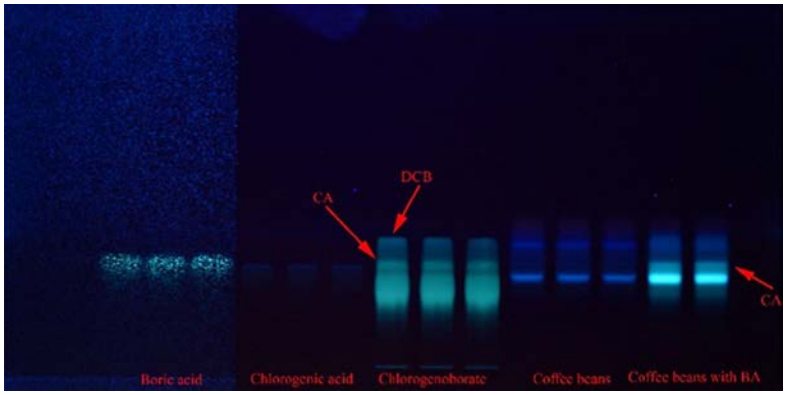

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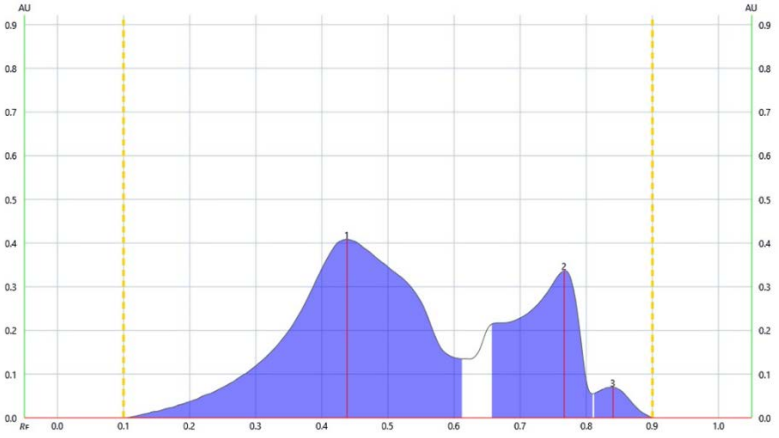

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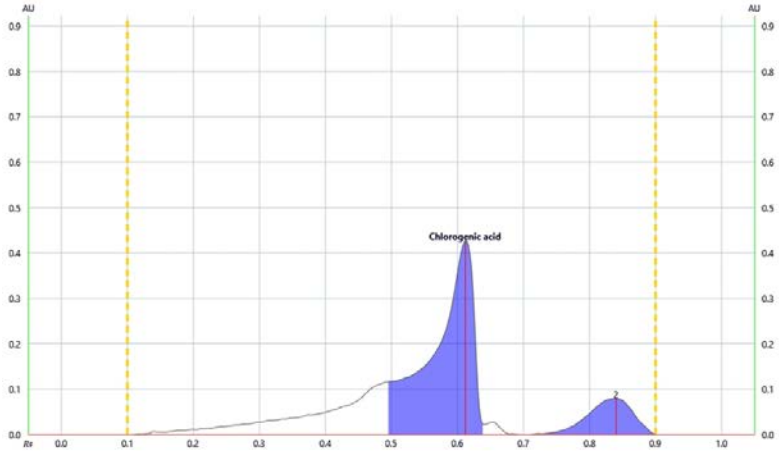

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Figure 1. HPTLC/UV densitometry: (a) HPTLC chromatogram at 254 nm; (b) HPTLC chromatogram at 365 nm; (c) UV densitogram for DCB complex (peak 2) at 280 nm; (d) UV densitogram for CA at 280 nm. In both 254 nm and 365 nm chromatograms, a new band was obtained for the chlorogenoborate sample and in the GCB extract with added BA, the band corresponding to the CA turns bright blue in 365 nm UV light. BA: Boric acid; CA: Chlorogenic acid; DCB: Diester chlorogenoborate; GCB: Green coffee bean; HPTLC: High-performance thin-layer chromatography; UV: Ultraviolet.

We identified the DCB peak in selective ion recording (SIR) mode, at 5.75 minutes, in a concentration of 2.48 μg/g of dry product (Figures 2–4, Table 1).

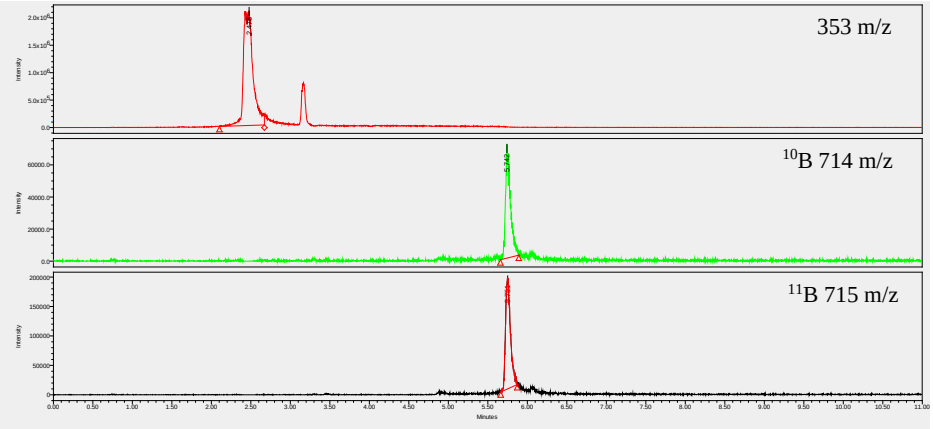
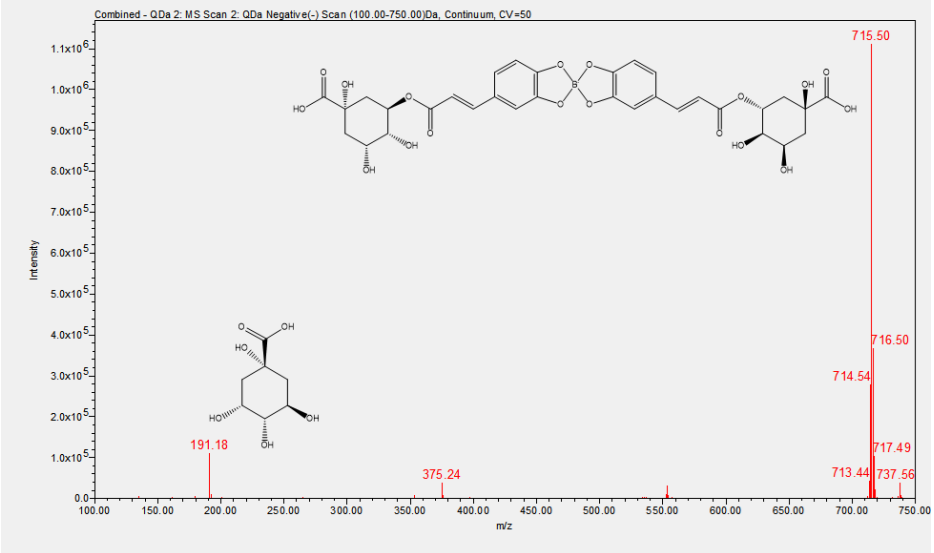


Figure 2. UHPLC/MS confirmation of the identified compounds. MS: Mass spectrometry; UHPLC: Ultra-high-performance liquid chromatography.

(a)



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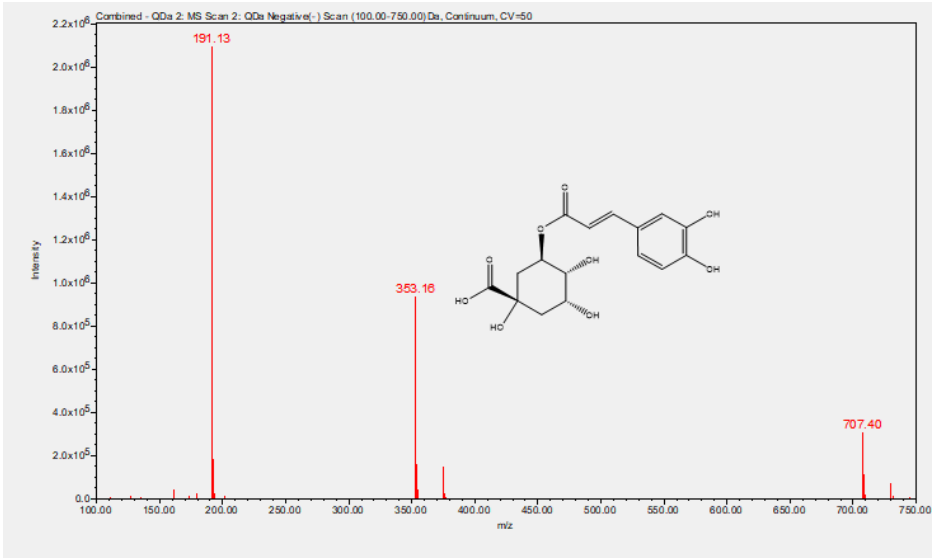

(b)

Figure 3. Mass spectrometry analysis: (a) Mass spectrum of DCB semisynthetic standard (fragment ion *m/z* 715) with quinic acid fragment ion (*m/z* 191); (b) Mass spectrum of CA (fragment ion *m/z* 353). CA: Chlorogenic acid; DCB: Diester chlorogenoborate.

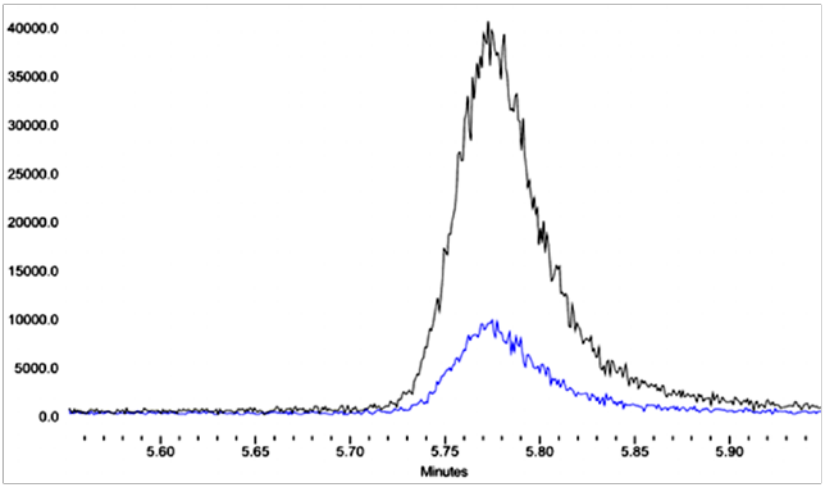

Figure 4. DCB complex identified in GCB extract. Blue chromatogram represents ^{10}B, while the black one highlights ^{11}B. The ratio between the areas is approximately 1:5, which is corresponding to the specific B isotope ratio. B: Boron; DCB: Diester chlorogenoborate; GCB: Green coffee bean.

Table 1. RTs and relative areas of the identified compounds.

Compound	RT [min]	Relative area
CA	2.478	20 921 574
DCB ^{10}B	5.742	173 776
DCB ^{11}B	5.742	818 884

B: Boron; CA: Chlorogenic acid; DCB: Diester chlorogenoborate; RT: Retention time.

The structure of DCB was proven by UV densitometry (Figure 1), mass spectrometry (Figures 1–4) analysis, FTIR spectroscopy (Figure 5), and the structural formula was proposed (Table 2). The coupling reaction was also discovered to be efficient at room temperature. DCB is readily separated from unreacted CA by TLC, ion exchange chromatography, or solvent extraction, as is well known in the art. As seen from the chromatograms, a relatively high amount of CA remains after the reaction and can be identified by the

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large peak at 2.50 minutes (Figure 2, Table 1). The newly formed compound can be observed at 5.75 minutes (Figure 2, Table 1).

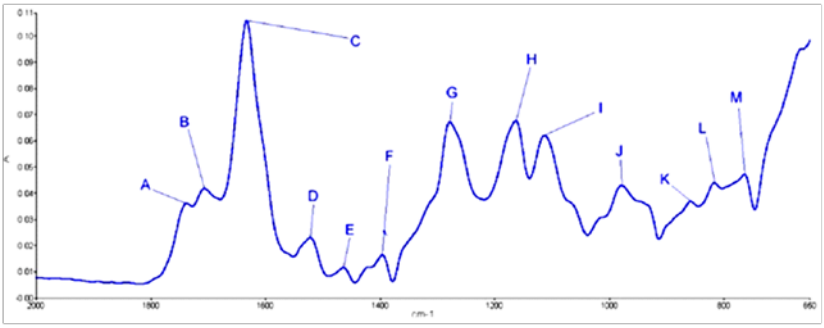
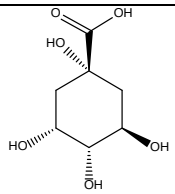
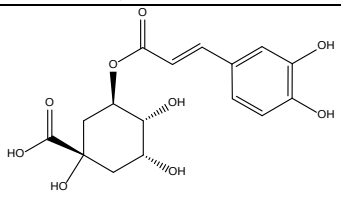
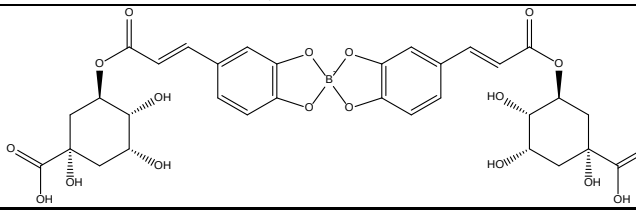


Figure 5. FTIR spectrum of DCB complex. Signed on IR spectrum: (A) α,β unsaturated aliphatic ester, C=O stretching of protonated carboxylic acid and some esters decompose (1737.51 cm^{-1}); (B) Some esters decompose (1706 cm^{-1}); (C) Presence of dimer of caffeic acid ester (1632.89 cm^{-1}); (D) C–C stretching (benzene moiety and acyclic chain) and in plane deformation modes or rocking mode for H–H bond (1521.59 cm^{-1}); (E) Presence of dimer of caffeic acid ester and >C=O stretching of carboxylic acid (1463.67 cm^{-1}); (F) Rotation of phenyl CH (1396 cm^{-1}); (G) Wagging modes of CH_2 group (1278.15 cm^{-1}); (H) C–O stretching band of triglycerides, could be assigned to the esters formation (1162.68 cm^{-1}); (I) C–O stretching band of triglycerides (1112.78 cm^{-1}); (J) N/A; (K) N/A; (L) B–O bond (816.69 cm^{-1}); (M) B–O bond (763.36 cm^{-1}). DCB: Diester chlorogenoborate; FTIR: Fourier-transform infrared (spectroscopy); N/A: Not assigned.

Table 2. Fragment ions (*m*/*z*) found in the mass spectra and supposed molecular structure for DCB complex.

Compound	Fragment ions (*m*/*z*)	Molecular structure
CA	191	 Quinic acid
	353	 CA
	707	CA dimer
DCB complex	191	Quinic acid
	715	

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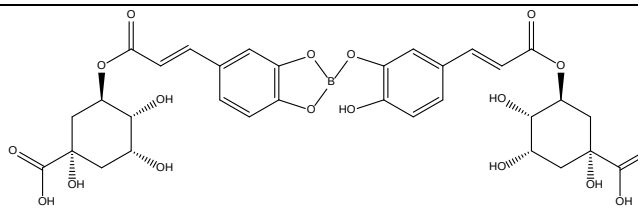
Compound	Fragment ions (*m*/*z*)	Molecular structure
		
		Newly formed DCB complex

CA: Chlorogenic acid; DCB: Diester chlorogenoborate.

CA has the specific fragmentation pattern, with *m*/*z* 191 $[M–H]^-$ for quinic acid, *m*/*z* 353 $[M–H]^-$ for CA and *m*/*z* 707 $[2M–H]^-$ (CA dimer). The newly formed compound exhibits the specific pattern for a B compound: 80% *m*/*z* 715 $[M–H]^-$ for ^{11}B and 20% *m*/*z* 714 $[M–H]^-$ for ^{10}B. For quantification purposes, the SIR for *m*/*z* 715 was used (Table 2).

In a gastric environment of pH 4.5 (after the meal), DCB is protected and is working as a classical prebiotic. The same effect is happening when pH 7.2 (similar with small intestine, the degradation rate is very low) (Figure 6).

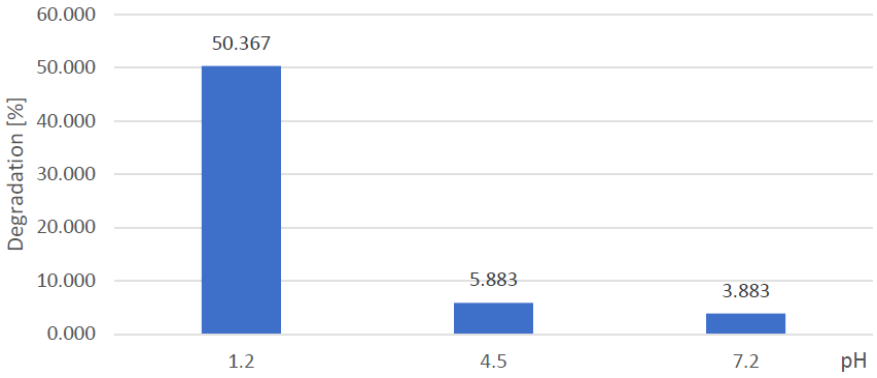


Figure 6. ***In vitro*** **simulation of DCB digestion:** stomach (gastric phase, at pH 1.2 and pH 4.5) and duodenum (small intestinal phase, at pH 7.2). DCB: Diester chlorogenoborate.

5. Discussion

The HPTLC assay uncovered a band at an R_f of 0.78, which corresponded to the fragment ion *m*/*z* 715. CA remained lower at an R_f of 0.45. The CA standard band appears higher, at around R_f of 0.61 due to the lower concentration (Figure 1).

The fragment ion *m*/*z* 715 also showed *m*/*z* 714 that was approximately 20% lower than the previous mentioned, indicating a B-containing compound (Figure 3a).

The DCB complex was also identified in GCB *via* UHPLC/MS technique (Figure 4). Extraction was achieved using 90% acetonitrile, which was then evaporated and resolubilized in the first line of the gradient. The same 1:5 B isotope ratio was observed.

Our previous studies demonstrated that using HPTLC/UV densitometry with confirmation by online HPTLC/ESI–MS, the identification of nicotinamide riboside and naturally occurring B (NOB) compounds is accurate, precise, rapid, selective, and sensitive [18, 24]. For the analysis of semisynthetic DCB complex and for the DCB identification in GCB, the previously developed methods were chosen (HPTLC/UV densitometry with confirmation by online HPTLC/ESI–MS) [18, 24] together with UHPLC/MS [22].

We chose only the plants that had a B concentration higher than 1 ppm and high polyphenols, to increase the possibility of identifying new B compounds (especially B-containing polyphenol compounds). After the rigorous tests previously reported, we chose only the GCB that had the highest amount of B organic species [23].

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DCB complex being a B phenolic compound can be considered as a prebiotic, acting as a vector both of B (an essential micronutrient for symbiosis) and CA [22]. CA has previously been established as a prebiotic phenolic acid that is only partially digestible in the gastric system [14].

Food grade DCB complex [25] is potentially useful for: (*i*) increasing of the buffering capacity of saliva; (*ii*) positively impacting the intestinal and oral microbiome; (*iii*) protecting important probiotic bacteria, *Bifidobacterium* spp. and *Lactobacillus* spp.; (*iv*) improving short-chain fatty acids (SCFAs) production; (*v*) improving intestinal barrier integrity and impermeability; (*vi*) improving the immunity system; (*vii*) developing psychobiotic products with nootropic effects; (*viii*) improving anti-inflammatory and antioxidant actions of the microbiota; (*ix*) providing chemical protection against radiation, in conditions of natural radioactivity in soil and water or/and nuclear disasters. Moreover, reagent grade DCB may be useful for: (*i*) reagent grade (≥95%) adequate for drug, food, or medicinal usage and appropriate for usage in many various analytical applications; (*ii*) some colon-targeted delivery systems for antioxidant therapy in the treatment of inflammatory bowel disease and B neutron capture therapy (^{10}BNCT) treatment of colon cancers by using ^{10}B-enriched prebiotic DCB complex [25, 26]. We were able to synthesize and identify DCB, and the uses of this new natural B complex were provisionally patented in the USA [25]. In the future, B phenolic complexes, such as DCB, may be promising new prebiotic candidates [22]. However, the latest investigation proves that in bacteria, the furanosyl borate diester [autoinducer-2 borate (AI-2B)] signaling molecule could contribute to the health of intestinal flora and its defense against bacteria pathogens [1, 2, 27]. New insights into B essentiality in human and animals show that a new concept in B science is needed to explain the mechanism of action of B in their health [22]. Given this new insight, the essentiality of B species has the potential to provide new opportunities for supplementing DCB complex in human/animal nutrition to support health and longevity [2, 22, 28, 29]. New knowledge about the essentiality of B species for a healthy symbiosis between human/animal host and microbiota may lead to the development of natural B-based nutraceuticals to target the human/animal microbiome (gut, oral, vaginal, skin, and scalp microbiome).

DCB complex is a novel prebiotic candidate, and it does not dissociate postprandially at pH 4.5 in the upper gastric system [22]. After completing all the phases of intestinal degradation, we will be able to draw the best conclusions on how to protect DCB complex in the upper gastric system to reach the colon intact, where it is essential and microbiota accessible. Further, studies show that B organic complexes are digested by microbiota [22, 26] and the dissociated phenolics and CA are further metabolized into functional compounds [16]. An increasing number of studies show the impact of nutrition on gut microbiota as well as the dependence of human health on healthy microbiota [30].

6. Conclusions

For the first time, we report that a new natural B organic complex, DCB, was obtained by semisynthesis and then discovered in plants (GCB). DCB may be a promising new prebiotic candidate to target the human/animal microbiome.

Author contributions:

IRS had the idea for the article. ABi, IRS, LEB, GR, CB, and LD performed the literature search and data analysis. ABi, IRS, MVC, ABu, and GDM designed and conducted the research. ABi, IRS, NR, JN, and GDM drafted and critically revised the work. IRS had primary responsibility for the final content. All authors read and approved the final manuscript.

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Conflict of Interest:

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical Approval:

The manuscript does not contain experiments on laboratory animals.

Consent to Participate:

The manuscript does not contain clinical studies or patient data.

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