Phenolic Profile and Antioxidant Activity of Chocolates Supplemented with Bioactive Ingredients

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Phenolic Profile and Antioxidant Activity of Chocolates Supplemented with Bioactive Ingredients

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Abstract

The growing demand for functional foods has stimulated the development of chocolate matrices enriched with bioactive ingredients. This study aimed to optimize the extraction of phenolic compounds using hydro-organic solvents of different polarities, characterize the phenolic and alkaloid profile of distinct chocolate formulations by HPLC-DAD, and evaluate whether fortification with microencapsulated vitamins E and D, zinc, and omega-3 fatty acids (EPA and DHA), either alone or combined with partial replacement of cocoa butter by a structuring oil, enhances the extractable bioactive compounds and in vitro antioxidant activity. Five formulations were evaluated: control chocolate (C), chocolate containing vitamin microcapsules (T1), chocolate with DHA/EPA microcapsules (T2), lipid-modified chocolate with structuring oil (T3), and chocolate combining microcapsules with lipid modification (T4). Phenolic compounds were extracted using 50% ethanol, 70% methanol, and 70% acetone. Among the tested solvents, 70% methanol showed the highest extraction efficiency, enabling broader detection of bioactive compounds. HPLC-DAD analysis revealed compounds characteristic of cocoa-based matrices, including epicatechin, gallic acid, vanillin, and traces of procyanidins, as well as the methylxanthine alkaloids theobromine and caffeine. The T4 formulation showed a greater abundance of extractable compounds compared to the other formulations. Antioxidant activity was evaluated using DPPH radical scavenging and β-carotene/linoleic acid bleaching assays. T4 exhibited the highest antioxidant performance, with 21.8% DPPH inhibition (EC_{50} = 2.5 mg/mL) and the highest optical density in the β-carotene assay, indicating enhanced protection against lipid peroxidation. These findings demonstrate that the combination of microencapsulation and lipid phase modification improves antioxidant functionality and supports the development of value-added functional chocolates.

Keywords: chocolate; phenolic compounds; antioxidant activity; bioactive ingredients

1. Introduction

The rising demand for functional foods has stimulated the development of food matrices enriched with added bioactive compounds capable of providing health benefits. In this context, chocolate stands out not only for its wide sensory acceptance and indulgent nature, but also for its intrinsic composition rich in naturally occurring phenolic compounds such as epicatechin, procyanidins, and gallic acid, which are recognized for their antioxidant, anti-inflammatory, and cardioprotective properties [1]. Due to its unique properties, chocolate can simultaneously act as a natural source of polyphenols and as a delivery system for microencapsulated lipophilic micronutrients, such as vitamins E and D, zinc, and the omega-3 fatty acids EPA and DHA, thereby expanding its potential as a functional food [1–3].

In Brazil, chocolate consumption is high and culturally widespread. Data from the *Pesquisa de Orçamentos Familiares* (POF 2017–2018), conducted by the Brazilian Institute of Geography and Statistics -IBGE, revealed that over 75% of Brazilian households consume chocolate products, mainly

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in industrialized forms such as bars, bonbons, and chocolate powders [4]. This widespread consumption highlights both the cultural and economic relevance of chocolate and its potential as a carrier for bioactive compounds, particularly in nutritional reformulation strategies [5].

However, the extractability and stability of chocolate polyphenols are strongly influenced by the food matrix. The lipid phase, predominantly composed of cocoa butter, plays a central role in the solubilization, protection, and release of these compounds during processing and throughout product shelf life [6,7]. For this reason, structural modifications of the lipid fraction, such as partial replacement with oils rich in bioactive fatty acids or structuring oleogel systems, have been investigated as strategies to preserve and enhance the functionality of phenolic compounds in chocolate and are also addressed in the present study [5,8,9].

Additionally, technologies such as microencapsulation of micronutrients, including fat-soluble vitamins and polyunsaturated fatty acids, have been widely studied as strategies to improve the oxidative and thermal stability of these ingredients and to modulate their release in food matrices, contributing to the preservation of their functional activity in the final product. In this context, the present study evaluates the impact of incorporating microencapsulated lipophilic micronutrients into a chocolate matrix on the extractable phenolic profile and *in vitro* antioxidant activity [10–12].

In this context, the characterization of phenolic compounds and the evaluation of antioxidant activity in chocolates are essential to support functional claims and potential preventive applications. Chromatographic methods, particularly high-performance liquid chromatography coupled with diode array detection (HPLC-DAD), provide high sensitivity and specificity in identifying these compounds, while complementary *in vitro* assays, such as DPPH radical scavenging and the *β*-carotene/linoleic acid system, yield relevant parameters regarding the biological functionality of the analyzed systems [5,13–15].

Therefore, this study aimed to compare and optimize the extraction of phenolic compounds and alkaloids from chocolates using hydro-organic solvents of different polarities, in order to maximize the recovery and detection of these compounds by HPLC-DAD; to qualitatively characterize the phenolic and alkaloid profiles of the different formulations; and to evaluate whether fortification with microencapsulated vitamins E and D, zinc, and the omega-3 fatty acids EPA and DHA, alone or in combination with lipid phase modification, increases the extractable bioactive compound content and in vitro antioxidant activity.

2. Materials and Methods

2.1. Preparation of Chocolate Formulations

For this study, five dark chocolate formulations were developed, each with a total cocoa content of approximately 60% (comprising cocoa liquor and cocoa butter), with modifications in the lipid phase composition and the inclusion of microencapsulated lipophilic micronutrients. Table 1 presents the percentage composition of the ingredients used in each sample. The raw materials included cocoa liquor and cocoa butter (IBC – Indústria Brasileira de Cacau, Brazil), refined sugar (União, Brazil), soy lecithin and polyglycerol polyricinoleate (PGPR) (Tovani, Brazil), powdered vanillin flavoring (Mix, Brazil), and hydroxypropylmethylcellulose (HPMC) (Metachem, Brazil). The samples were designated as follows: C (Control), standard chocolate without microcapsules or lipid replacement; T1, chocolate with microcapsules containing vitamins E, D, and zinc; T2, chocolate with microcapsules containing polyunsaturated fatty acids (DHA and EPA) and high-oleic peanut oil incorporated into the lipid phase; T3, chocolate with 30% of cocoa butter replaced by a structuring oleogel composed of 47% (w/w) Brazil nut oil, 1.5% (w/w) HPMC, and distilled water (as detailed in section 2.3); and T4, chocolate with structuring oleogel combined with both types of microcapsules (vitamins E, D, and zinc; DHA and EPA with high-oleic peanut oil).

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Table 1. Percentage composition of ingredients in chocolate formulations (dry basis).

Ingredients %	C	T1	T2	T3	T4
Cocoa liquor	50.0	50.0	50.0	53.0	53.0
Cocoa butter	10.0	10.0	10.0	7.0	7.0
Refined sugar	39.2	39.1	29.0	26.2	26.1
Soy lecithin	0.5	0.5	0.5	0.5	0.5
PGPR	0.1	0.1	0.1	0.1	0.1
Vanillin	0.2	0.2	0.2	0.2	0.2
Microcapsules (E/D/Zn)	–	10.0	–	–	5.0
Microcapsules (DHA/EPA + óleo)	–	–	10.0	–	5.0
Oleogel	–	–	–	3.0	3.0

The amount of cocoa butter used in the samples were calculated to ensure that the final product would have a lipid content of 35%, thereby composing a standard dark chocolate with 60% cocoa solids.

2.2. Chocolate Production

The production of the samples was carried out at the Food Technology Laboratory III of the Faculty of Pharmaceutical Sciences, University of São Paulo. Manufacturing was performed using a universal mixer with a ball mill, model WA-FA20 (Mazzetti, Italy), which integrates the processes of mixing, refining, and conching into a single system, thereby optimizing space, time, and energy. The ingredients were sequentially added to the equipment at 45 °C. After processing, the chocolates were manually tempered on a marble slab, molded into appropriate forms, and cooled at 5 ± 3 °C for 20 minutes. Subsequently, they were demolded and stored for further analysis.

2.3. Oleogel Preparation

The oleogel was prepared according to the method described by Espert et al [35], with adaptations by Santos, Suzuki and Lannes [5]. The emulsion consisted of 47% (w/w) Brazil nut oil, 1.5% (w/w) HPMC, and distilled water to complete 100% (w/w). Initially, HPMC was dispersed in the oil under mechanical stirring (Fisaton, Brazil) at 280 rpm for 5 minutes. Subsequently, chilled water (10 °C) was added to promote cellulose hydration, and the mixture was homogenized using an Ultra-Turrax (Marconi, Brazil), disperser S18N-19G, first at 6,500 rpm for 1 minute and then at 17,500 rpm for 3 minutes. The resulting white, viscous emulsion was poured into aluminum trays (40 × 20 cm) and dried in an adiabatic oven at 60 °C for approximately 48 hours until a moisture content below 5% was reached.

2.4. Extraction of Polyphenols and Alkaloids

The extraction of phenolic compounds and alkaloids was carried out based on the method of Sánchez-Rabaneda et al. [22] with modifications. Chocolate samples (5 g), previously ground (300 µm), were defatted with 40 mL of hexane under agitation for 2 minutes (Ultra-Turrax Ystral,

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Germany). After centrifugation at 500 rpm for 5 minutes at 15 °C (Sigma 3-16K centrifuge, Germany), the solid residue was used for extraction.

The extraction was performed using three hydro-organic solvents of different polarities to optimize the recovery of phenolic compounds with varying polarities: 70% methanol (70Me) (polarity index: 0.762), 50% ethanol (50Et) (polarity index: 0.654), and 70% acetone (70Ac) (polarity index: 0.355), representing a range from moderate to low polarity in water–organic systems. For each solvent, 30 mL were added to the pellet, followed by vortex mixing (Velp Scientifica ZX3, Italy) for 3 minutes and sonication in an ultrasonic bath (Bandelin Sonorex RK510S, Germany) for 10 minutes at 25 °C. The samples were centrifuged again under the same conditions, and the supernatants were collected.

This procedure was repeated twice with the same residue. The obtained extracts were combined and concentrated under reduced pressure in a rotary evaporator (R-300, Büchi, Switzerland). The dried residue (approximately 30 ± 5 mg) was reconstituted in 10 mL of methanol, filtered through a 0.45 μm membrane, and diluted 1:10 in methanol:water (50:50, v/v) prior to HPLC analysis.

2.5. HPLC-DAD Analysis of Polyphenols and Alkaloids

Polyphenol and alkaloid profiling was carried out using a high-performance liquid chromatography system coupled to a diode array detector (HPLC-DAD; Thermo Scientific™ Vanquish, model VC-D11-A), equipped with a C18 UHPLC column (Vanquish™), maintained at 25 °C. The mobile phase consisted of water containing 0.1% formic acid (Phase A) and methanol containing 0.1% formic acid (Phase B). The acidic mobile phase system was selected to enhance chromatographic separation and peak definition of phenolic compounds across a wide polarity range.

The elution gradient started at 95% Phase A and 5% Phase B, reaching 70% A and 30% B within 5 min. At 24 min, the system achieved 100% Phase B and was returned to the initial conditions at 30 min to ensure column re-equilibration. The flow rate was set at 1.0 mL/min, and the injection volume was 10 μL. This gradient program allowed the sequential elution of more polar phenolic acids followed by semi-polar flavonoids and more lipophilic compounds, providing a representative chromatographic fingerprint of cocoa-derived phenolics.

Compound identification was performed based on retention time (RT) and UV–Vis spectral characteristics obtained from the diode array detector, compared with commercial analytical standards (epicatechin, gallic acid, and procyanidin B2; Sigma-Aldrich, St. Louis, MO, USA) analyzed under identical chromatographic conditions. Detection wavelengths were selected according to the characteristic absorption maxima of the target compounds, namely 280 nm for catechin, epicatechin, and procyanidins, and 270 nm for gallic acid.

The HPLC-DAD analysis was conducted for qualitative and comparative purposes, aiming to characterize the extractable phenolic profile and evaluate differences among chocolate formulations. Relative comparisons between samples were based on chromatographic profiles and peak area distribution, allowing the assessment of formulation-dependent effects on phenolic extractability. Data acquisition and processing were performed using Chromeleon™ Data System software, with peak integration carried out consistently across all samples to ensure comparability of the chromatographic fingerprints.

2.6. Determination of Antioxidant Capacity

2.6.1. DPPH Radical Scavenging Assay

Antioxidant capacity was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical scavenging method, adapted from Moure *et al.* [16]. Samples were diluted to 0.5 mg/mL; 50 μL of each sample were mixed with 2 mL of a methanolic DPPH• solution (14.2 μg/mL). The reaction mixture was maintained in the dark for 30 min, and absorbance was measured at 517 nm using a Hitachi U-3900 spectrophotometer. Results were expressed as inhibition percentage (IP%), Trolox

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equivalents (μg TE/mL), and EC50 (mg/mL), defined as the concentration required to inhibit 50% of DPPH radical.

2.6.2. β-Carotene Bleaching Assay

The assay followed Miller [17] with modifications. An antioxidant emulsion was prepared with 2 mg *β*-carotene dissolved in chloroform, 40 mg linoleic acid, 400 mg Tween® 40, and 100 mL oxygenated water, homogenized and evaporated at 40 °C. Samples (0.2 mL at 5 mg/mL) were added to 5 mL of emulsion and incubated at 50 °C for 2 h. Absorbance was recorded at 380 nm at 0 and 120 min. Antioxidant activity was expressed as the Antioxidant Activity Coefficient (AAC), calculated from the absorbance difference between sample and control.

3. Results

Seventy percent methanol demonstrated the widest detection range: 18 of the 19 monitored compounds appeared in at least four of the five formulations (Table 2). The chromatographic profile revealed a dense array of peaks between 3 and 18 min, in addition to several late-eluting peaks (> 22 min). This result confirms the long-standing use of hydro-methanolic solvents as the gold standard for extracting polar and semi-polar phytochemicals [18,19].

Table 2. – Phenolic compounds and alkaloids identified by HPLC-DAD in chocolate samples extracted with 70% methanol.

Compounds	C	T1	T2	T3	T4
Vanillin	✓	✓	✓		✓
Vanillic acid	✓	✓	✓	✓	
Gallic acid	✓	✓	✓	✓	✓
Syringic acid	✓	✓	✓	✓	✓
p-Coumaric acid	✓	✓	✓	✓	✓
Caffeic acid	✓	✓	✓	✓	✓
Ferulic acid	✓	✓	✓	✓	✓
Luteolin	✓		✓	✓	✓
Quercetin	✓	✓	✓	✓	✓
Kaempferol	✓	✓		✓	✓
Epicatechin	✓	✓	✓	✓	✓
Epigallocatechin	✓	✓	✓	✓	✓
Caffeine	✓	✓	✓	✓	✓
Theophylline	✓	✓			
Theobromine	✓	✓	✓	✓	✓

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Seventy percent methanol demonstrated the widest detection range: 18 of the 19 monitored compounds appeared in at least four of the five formulations (Table 2). The chromatographic profile revealed a dense array of peaks between 3 and 18 min, indicating the presence of several polar and semi-polar compounds in the extracts. These findings reinforce the well-established use of hydro-methanolic solvents as effective extractants for a wide range of polar and semi-polar phytocomplexes [18,19].

Table 3. – Phenolic compounds and alkaloids identified by HPLC-DAD in chocolate samples extracted with 50% ethanol.

Compounds	**C**	**T1**	**T2**	**T3**	**T4**
Vanillin	✓				
Vanillic acid	✓	✓			
Gallic acid	✓	✓	✓	✓	
Syringic acid	✓	✓		✓	✓
p-Coumaric acid	✓				✓
Caffeic acid	✓	✓		✓	✓
Ferulic acid	✓	✓	✓		
Luteolin	✓	✓			
Quercetin	✓		✓		
Kaempferol	✓				
Epicatechin	✓	✓		✓	
Epigallocatechin				✓	
Caffeine					
Theophylline	✓				
Theobromine ✓	✓	✓	✓	✓	

In **50% ethanol (Table 3)**, a reduction in the number of detected compounds was observed compared with the methanolic extraction. In addition, several peaks present in the methanolic extract were not detected under the ethanolic extraction conditions. This difference may be related to

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variations in extraction efficiency between solvent systems, particularly considering the influence of solvent polarity on the solubilization of phenolic compounds from complex food matrices [20].

Table 4. – Phenolic compounds and alkaloids identified by HPLC-DAD in chocolate samples extracted with 70% acetone.

Compounds	C	T1	T2	T3	T4
Vanillin	✓	✓			
Vanillic acid					
Gallic acid	✓				
Syringic acid					
p-Coumaric acid					
Caffeic acid					✓
Ferulic acid	✓				
Luteolin					
Quercetin					
Kaempferol					
Epicatechin	✓				
Epigallocatechin					
Caffeine					
Theophylline					
Theobromine ✓	✓	✓	✓	✓	

Ac70 (Table 4) showed poor extraction performance, with few compounds detected and poorly resolved peaks. Its low polarity and aprotic character limit the extraction of phenolics in fat-rich matrices such as chocolate. Although useful for dried plant materials, its effectiveness is reduced in lipophilic and structured systems, such as formulations containing oleogel or microcapsules, and therefore its use should be avoided or carefully adjusted [18–20].

	70% Me	50% Et	70% Ac

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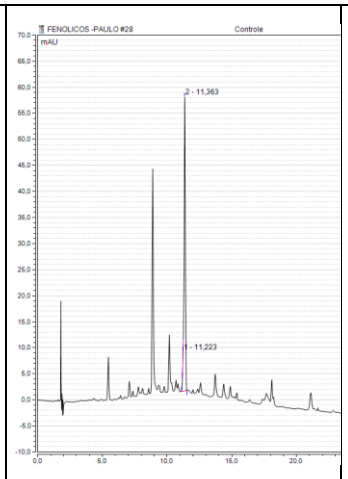
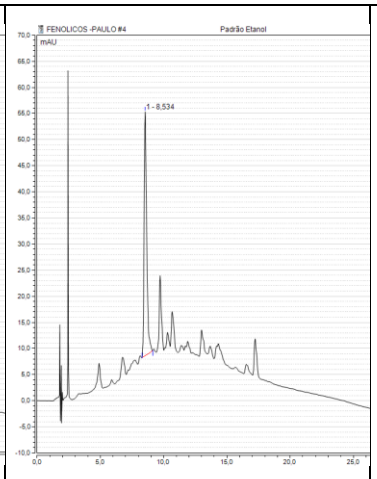
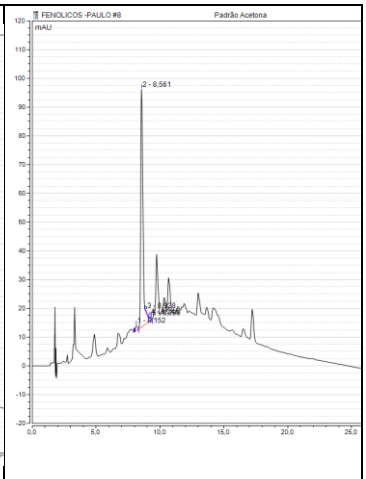
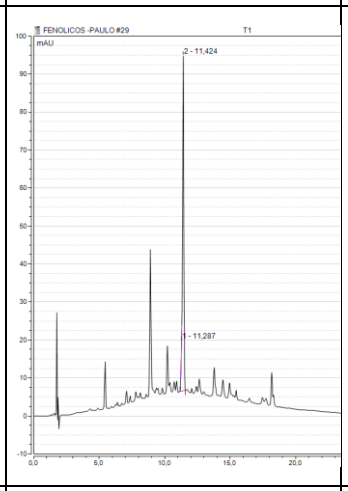
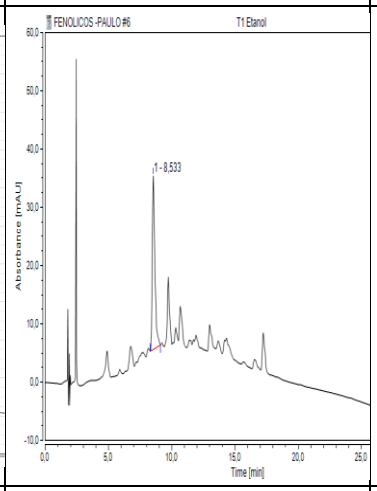
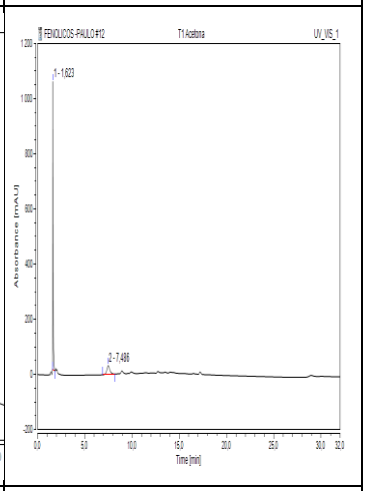
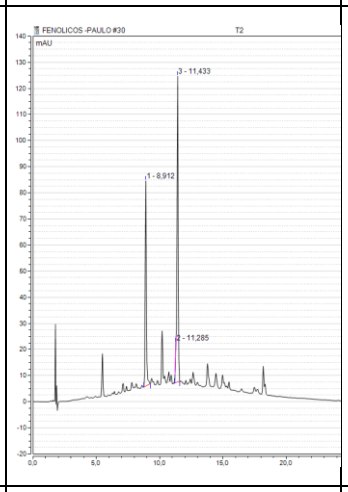
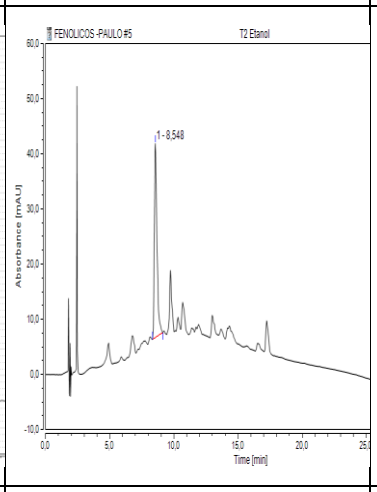
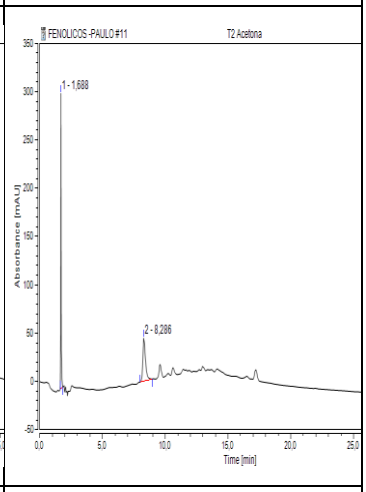
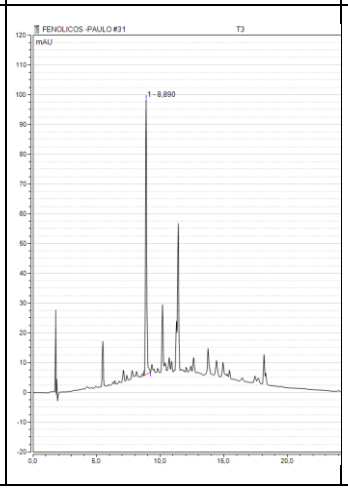
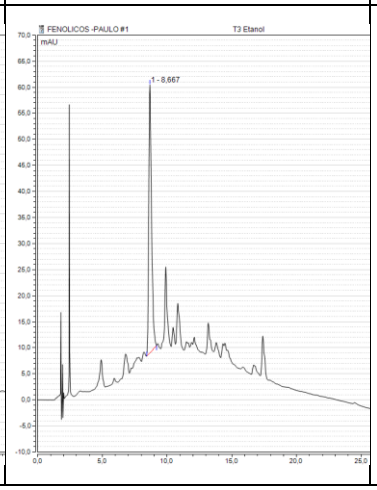
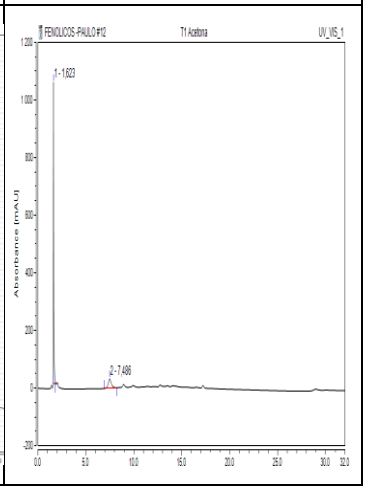
C			
T1			
T2			
T3			

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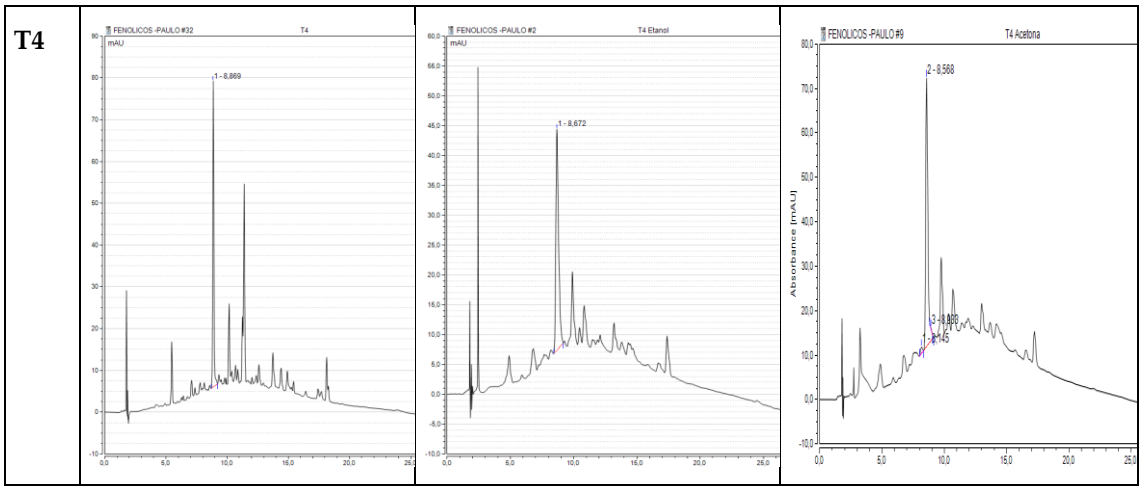

Figure 1. – HPLC-DAD chromatograms (detection at 280 nm) of chocolate extracts obtained with different solvents (70% methanol, 50% ethanol, and 70% acetone). Samples: C (control), T1 (vitamin D, E and zinc microcapsules), T2 (DHA/EPA), T3 (oleogel), T4 (microcapsules + oleogel).

Figure 1 visually synthesizes the impacts of solvent choice and matrix reformulation on the chromatographic profiles obtained by HPLC-DAD. It can be noted that methanolic extracts produced the densest traces, with multiple well-defined peaks across the entire retention range, especially between 4 and 18 minutes. This distribution is characteristic of extracts rich in phenolic acids, flavan-3-ols, flavonols, and methylxanthine alkaloids, whose solubility is favored by highly polar and protic solvents such as methanol [15,18]. In contrast, the chromatograms obtained with ethanol and acetone displayed lower complexity and a significant reduction in signal intensity in critical regions, indicating reduced accessibility and extraction of certain phenolic compounds. Such losses are related both to the physicochemical properties of the solvents and to structural modifications introduced by chocolate matrix reformulations, which interfere with the release and solubilization of the compounds [20].

Table 5. – Distribution of phenolic compound classes and alkaloids across formulations and extraction solvents (70% Me, 50% Et, 70% Ac), determined by HPLC-DAD. Data reflects combined effects of compound polarity, matrix accessibility, and solvent-matrix interactions. RT, retention time (minutes).

Class (RT)	Main Evidence	Discussion	References
Hydroxybenzoic acids (4–7 min)	Peaks detected mainly in methanolic extracts; fewer signals observed in ethanol and acetone.	These compounds present relatively high polarity due to hydroxyl and carboxyl groups, which favors extraction with hydroalcoholic solvents.	18, 19, 20
Hydroxycinnamic acids (7–9 min)	Signals observed mainly in methanolic	The conjugated aromatic structure influences retention	19, 20

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	extracts, with reduced recovery in ethanol and acetone systems.	behavior and extraction efficiency depending on solvent polarity.	
Flavan-3-ols / procyanidins (9–14 min)	Peaks predominantly observed in methanolic extracts.	Multiple hydroxyl groups increase polarity, favoring extraction in more polar solvent systems.	20, 21
Benzaldehyde derivatives (e.g., vanillin) (≈10–12 min	Peaks corresponding to aromatic aldehyde derivatives detected in the chromatographic profile.	These compounds are common aroma-related phenolics in cocoa matrices and may appear in intermediate retention regions.	22
Flavonols / flavones (14–18 min)	Signals mainly detected in methanol extracts and less frequently in ethanol extracts.	Polyhydroxylated aromatic structures generally require polar solvents for efficient extraction.	15, 20
Alkaloids (3–5 min)	Early peaks consistent with methylxanthine-type compounds detected in the samples.	Cocoa naturally contains methylxanthines, which are stable compounds commonly observed in chromatographic analyses of cocoa products.	23

The class-based analysis of compounds (Table 5) provides a clearer understanding of extraction patterns and the underlying chemical mechanisms. Phenolic acids (RT 4–9 min), such as gallic, caffeic, and ferulic acids, showed higher extractability in methanolic systems, intermediate performance with ethanol, and were almost completely absent in acetonic extracts. This behavior confirms that the high density of free hydroxyl groups in these compounds requires highly polar, protic environments to promote ionic dissociation and stabilization of anionized species, a phenomenon not favored in aprotic solvents such as acetone [18–20].

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Antioxidant activity

Table 6 presents the antioxidant activity of the different treatments evaluated using the DPPH radical scavenging assay and the β-carotene bleaching method. Significant differences were observed among the formulations ($p < 0.05$), with treatments T3 and T4 showing higher antioxidant activity compared with the control and the other formulations. These results indicate variations in the antioxidant potential among the samples, as reflected by both analytical methods. The detailed discussion of these findings and their relationship with the formulation composition is presented in the following section.

Table 6. – Antioxidant Activity.

Treatment	DPPH (μmol TE g^{-1})	β-Carotene bleaching (%)
Control	35.9 ± 1.0 [a]	40.0 ± 0.9 [a]
T1	38.5 ± 1.2 [a]	42.1 ± 1.4 [a]
T2	39.4 ± 0.9 [a]	43.3 ± 1.1 [a]
T3	47.1 ± 1.1 [b]	54.2 ± 1.2 [b]
T4	55.7 ± 1.3 [c]	64.9 ± 1.2 [c]

Identical letters in the column indicate no significant difference (Tukey, $p > 0.05$).

4. Discussion

4.1. Phenolic Profile

The qualitative evaluation of methanolic, ethanolic, and acetonic extracts obtained from the five chocolate formulations (C, T1–T4) revealed marked differences in the ability of each solvent to solubilize phenolic compounds and alkaloids. The detailed presence/absence data shown in Tables 2 to 4—complemented by the summary in Table 4 and the chromatograms in Figure 1—provide a comprehensive overview of the selectivity of each solvent, the effect of structural and compositional changes in the chocolate matrix on phenolic solubilization, and the consistency of the findings with contemporary literature.

First, the Me70 extract stood out by enabling the detection of virtually all monitored analytes. This performance can be associated with the high polarity of hydro-methanolic systems, which favor the extraction of phenolic compounds commonly present in cocoa matrices [18,19]. The chromatographic response (Figure 1) displayed a dense array of peaks between 3 and 18 min, indicating the presence of several compounds with different polarities extracted under these conditions. These findings highlight the suitability of hydro-methanolic solvents for recovering a wide range of phenolic compounds from cocoa-based products [18,19]. In contrast, the Et50 extract showed a marked reduction in the number and intensity of peaks, suggesting lower extraction efficiency compared with the methanolic system [20]. On the other hand, Ac70 proved inadequate for the chocolate matrix: only eight compounds were detected, with broad and poorly resolved peaks, which may indicate co-extraction of interfering lipids and reduced compatibility with polyhydroxylated phytochemicals present in cocoa matrices [19,20].

Beyond solvent composition, the structural characteristics of the chocolate matrix may also influence the extractability of phytochemicals. Formulations containing oleogel (T3) exhibited differences in the chromatographic profile compared with the control formulation, which may be related to modifications in the lipid network organization introduced by the oleogel system. Such structural changes can alter the interaction between phenolic compounds and the lipid phase, potentially affecting their release during solvent extraction [6]. In formulations containing vitamin microcapsules (T1) or DHA/EPA microcapsules (T2), variations in the chromatographic profiles were also observed, suggesting that encapsulating matrices may influence the accessibility of phenolic compounds. Polysaccharide-based encapsulating systems, such as those used in T1, may interact differently with polar phytochemicals compared with lipid–protein microcapsules such as those

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present in T2, which can modify the partition behavior of these compounds during extraction [21]. The hybrid formulation (T4), combining oleogel and microencapsulation strategies, presented a complex chromatographic profile among the reformulated samples, reinforcing the importance of considering matrix–compound interactions when evaluating functional chocolates developed with multiple technological approaches.

These results illustrate how the solvent–matrix interaction influences extraction efficiency in functional chocolates. Simple ingredient substitutions—such as the introduction of oleogels or microcapsules—alter the spatial distribution and solubility of phenolic compounds within the matrix, directly impacting HPLC profiles. This observation highlights the importance of considering structural modifications when developing extraction protocols for reformulated chocolate systems, particularly when functional or nutritional claims are assessed.[6,9]. Recent studies indicate that encapsulating structures or lipid networks can significantly modify diffusional behavior and intermolecular interactions of phenolics within the matrix, requiring extraction methodologies tailored to each case [19,20]. Therefore, qualitative analyses that overlook these structural modifications may underestimate or overestimate the presence of certain phytochemicals, compromising functional or nutritional interpretations. Such findings underscore the importance of integrated analytical strategies adapted to the physicochemical characteristics of reformulated systems, particularly in products with functional claims and nutraceutical appeal [18].

Differences among the formulations C (control), T1 (vitamin D, E and zinc microcapsules), T2 (DHA/EPA microcapsules), T3 (oleogel), and T4 (microcapsules + oleogel) were clearly reflected in the HPLC-DAD chromatographic profiles, revealing that matrix structural modifications directly affect the accessibility and selective extraction of phenolic compounds across solvent systems.

Formulation T2 (DHA/EPA microcapsules) showed a noticeable reduction in the number and intensity of peaks in the early chromatographic region (approximately 4–9 min), typically associated with phenolic acids, when compared with the control formulation in both methanolic and ethanolic extracts. This pattern suggests that the presence of lipid-rich microcapsules may influence the accessibility of certain polar phenolic compounds during solvent extraction, an effect that became more evident when less polar solvent systems were used, where a further decrease in detectable peaks was observed. In contrast, formulation T3 (oleogel) presented chromatographic profiles more similar to the control when polar solvents were applied, particularly within retention time regions associated with phenolic compounds, suggesting that structural modifications in the lipid phase may influence the release of these compounds during extraction. However, this tendency was less evident in less polar solvents. Additionally, signals consistent with aromatic aldehyde derivatives, such as vanillin, were observed in the chromatographic profile, indicating the presence of other phenolic-related compounds commonly found in cocoa matrices [2,5].

Formulation T4 (microcapsules + oleogel combination) exhibited synergistic restoration of extract complexity across all solvent systems, with chromatographic profiles approaching control levels in both methanol (↓5–10% density reduction) and ethanol (↓20% reduction), and remarkably, achieving nearly control-level recovery even in acetone (↓6% positional shift). The phenolic acids (2–8 min region) were restored with high intensity in T4, particularly in methanolic extracts, and the overall peak distribution recovered near-complete diversity. This indicates that the combined structural matrix effect of microcapsules and oleogel overcomes the individual limitations of each component, enabling solvent access to the full spectrum of phenolic compounds regardless of their polarity [6].

These results highlight the inherent complexity of extracting bioactive compounds in modified food matrices, emphasizing the importance of selecting solvents compatible not only with the chemical nature of the compounds but also with the physico-structural characteristics of the studied matrix. Moreover, the interaction between matrix components and encapsulating or structuring agents can directly influence the potential bioaccessibility and release of functional compounds in developed foods, a crucial aspect for the formulation of optimized functional products. Thus, the detailed analysis by chemical class and retention time, presented in Table 5, becomes essential to

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understanding the combined influence of solvent polarity and matrix structure on bioactive extraction and will be explored below.

Flavan-3-ols, represented by epicatechin (RT 9–14 min), were detected mainly in methanolic extracts, while absent in acetone extractions and partially compromised in ethanol, especially in sample T2. The absence of catechin in T2-Et is attributed to possible complexation with the DHA/EPA encapsulating matrix, which inhibits its diffusion into the extractive medium [20,21]. Moreover, the high degree of hydroxylation of these molecules reinforces the need for highly polar solvents with strong hydrogen-bonding capacity.

The class of flavonols and flavones (RT 14–18 min), including quercetin, kaempferol, and luteolin, demonstrated an even stronger dependence on solvent type and matrix modification. These compounds possess multihydroxylated aromatic structures and high dipole moments, making them better extracted with methanol. In ethanol, only quercetin was detected in T3, suggesting that the oleogel contributes to greater release of these molecules, possibly by promoting higher porosity of the lipid matrix and facilitating diffusion [15,20]. The complete absence of these classes in acetonic extracts once again highlights the limitations of this solvent in fatty systems.

Finally, compounds eluting in the late chromatographic region (RT > 22 min), together with early signals between 3 and 5 min, complete the overall chromatographic profile observed for the chocolate samples. The late retention region likely corresponds to compounds with lower polarity or higher molecular weight present in the cocoa matrix, whereas early signals are typically associated with more polar compounds that exhibit greater chromatographic mobility. Among the compounds commonly reported in cocoa-based products, methylxanthine-type alkaloids are known to occur and are characterized by their high stability and diffusibility in food matrices. These observations highlight that the extraction and detection of bioactive compounds are strongly influenced not only by the chemical characteristics of the solutes, but also by the physicochemical structure of the chocolate matrix and the solvent system used during extraction [23].

4.2. Antioxidant activity and influence of the lipid matrix

The antioxidant capacity of the five chocolate formulations—Control, T1 (vitamin E, D, and Zn microcapsules), T2 (DHA/EPA microcapsules), T3 (oleogel), and T4 (oleogel + microcapsules)—was evaluated using the DPPH• radical scavenging assay (μmol TE g^{-1}) and the β-carotene bleaching assay (inhibition %) (Table 6). The protocols followed Brand-Williams, Cuvelier, and Berset [24] for DPPH and Deghima et al. [25]. for β-carotene, complementary methods that measure, respectively, electron transfer and hydrogen transfer, thus providing a comprehensive view of both hydrophilic and lipophilic compounds [26,27].

According to studies by Todorovic et all [23]. milk chocolates rarely exceed 40 μmol TE g^{-1}, a value consistent with the control in this study. The addition of isolated microcapsules (T1 and T2) increased DPPH by less than 10%, a result similar to that observed by Tipaldi [28] who attributed the modest gain to the diffusional barrier imposed by the polymeric wall; however, after *in vitro* digestion, the activity may increase by up to 150% [29]. Lipid reformulation with oleogel (T3) raised DPPH by 31% and doubled β-carotene inhibition—an effect associated with the porous crystalline network that protects polyphenols and tocotrienols during conching [30,31]. The best performance was achieved with the hybrid system (T4), whose 55.7 μmol TE g^{-1} approximates the lower range of 70% cocoa chocolates; the synergy arises from the lipophilic bridge created by the oleogel, which facilitates the diffusion of compounds released from the microcapsules [32] and enhances protection against peroxyl radicals, as described by Medina Mendoza et al [33].

The strong correlation obtained between DPPH and *β*-carotene ($r \approx 0.9$), previously reported for cocoa products by Prior et al. [27], confirms that the increase in antioxidant activity simultaneously affects both electron- and hydrogen-transfer mechanisms. However, the *β*-carotene assay proved more sensitive to matrix modifications, reinforcing the decisive contribution of lipophilic antioxidants stabilized within the oleogel, such as carotenoids and tocopherols [26,34]. These results highlight that the concomitant engineering of the lipid phase and microencapsulation is an effective

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strategy to enhance immediate antioxidant potency without increasing cocoa content, aligning with the demand for functional chocolates with clean labeling.

5. Conclusions

The present study demonstrated that the combination of extraction solvents with different polarities and structural modifications of the lipid matrix significantly influences the recovery of phenolic compounds and the antioxidant activity of chocolates supplemented with bionutrients. Extraction with 70% methanol provided the broadest detection of bioactive compounds, confirming its efficiency in solubilizing polar and semi-polar substances. Among the formulations, chocolate containing both oleogel and microcapsules (T4) exhibited the most complete chromatographic profile and the highest antioxidant activity values, demonstrating the effective structural preservation and enhanced accessibility afforded by the combined technological strategies adopted.

Microencapsulation contributed to the stabilization and protection of micronutrients, while oleogel facilitated the diffusion of lipophilic and higher-molecular-weight compounds, promoting more efficient release within the food matrix. The data obtained support the potential of combining these technologies in the development of functional chocolates with enhanced antioxidant properties, without the need to increase cocoa content or add artificial ingredients.

For future research, absolute quantification of phenolic compounds using HPLC techniques coupled with mass spectrometry is recommended, in addition to assessments of bioaccessibility and bioactivity *in vitro* and *in vivo* digestion models. It is also important to investigate compound stability during storage and the sensory impact of reformulations, in order to ensure technological feasibility and consumer acceptance of the final product. These developments may consolidate functional chocolate as a high value-added food, with health benefits and aligned with trends of clean innovation and nutritionally strategic design.

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