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Optimization of Extraction and Drying Methods of Low-Grade Mulberry Leaf Tea Extract for Encapsulation in Alginate-Pea Protein Hydrogels with pH-Responsive Release Behavior

Jariyawan Pohkhet¹
Kamonnat Charoensri¹
Jutamane Sornnuwat¹
Chana Panyanon²,
Nut Thephuttee¹,
Tarit Apisittiwong³
Neeracha Sangpreecha^{1, 4}
Janisada Wattanasan¹
Phacharapan Santudprom¹,
Pitchaya Pothinuch^{1, *},

¹Faculty of Food Technology, Rangsit University, Pathum Thani 12000, Thailand

²Department of Chemistry, Faculty of Science, Rangsit University, Pathum Thani 12000, Thailand

³Department of Product Development, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand

⁴Food Processing Pilot Plant, College of Agricultural Innovation and Food Technology, Rangsit University, Pathum Thani 12000, Thailand

*Corresponding author; E-mail: pitchaya.p@rsu.ac.th

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Abstract

This study investigated alginate-pea protein hydrogel beads as an encapsulation material for antioxidants derived from low-grade mulberry leaf tea residue (LGMLT). The quality of LGMLT extract powder was significantly influenced by both extraction and drying methods with freeze drying providing greater retention of phenolic compounds and antioxidant activity than spray drying. Ultrasonication-assisted extraction combined with freeze drying gave the highest antioxidant activity (ABTS: 37.12 mg of Trolox equivalents/g and FRAP: 43.90 mg of Trolox equivalents/g) and a total phenolic content (22.97 mg gallic acid equivalents/g) comparable to that of decoction-freeze-drying, while spray drying consistently resulted in higher powder yield across extraction methods. The physicochemical and antioxidant properties of hydrogel beads were strongly dependent on the alginate-to-LGMLT extract ratio; higher alginate content improved bead uniformity, encapsulation efficiency (95.90%), and water-holding capacity (55.94%), whereas increased extract loading enhanced total phenolic content and antioxidant activity. *In vitro* pH-responsive release studies demonstrated a rapid release of phenolic compounds under pH 1.2, followed by a more sustained release at pH 7.4, reflecting the pH-dependent behavior of the hydrogel matrix. Differences between ABTS and FRAP responses indicated assay-dependent antioxidant capacities influenced by hydrogel composition. Based on these findings, alginate-pea protein hydrogels represent a tunable, food-grade encapsulation system for antioxidants and offer an alternative approach for the valorization of tea-processing residues in functional food applications, contributing to sustainable food production and food security.

Keywords: Alginate-pea protein hydrogel, encapsulation efficiency, phenolic compound encapsulation, mulberry leaf tea residue, phenolic compounds, pH responsive release

1. Introduction

In recent decades, shifts toward processed and energy-dense diets, together with environmental and lifestyle-related stressors, have contributed to an imbalance between reactive oxygen species (ROS) generation and the antioxidant defense systems of the human body. Excessive accumulation of ROS leads to oxidative stress, which is widely recognized as a key factor in the development of non-communicable diseases (NCDs), including cardiovascular diseases, diabetes, cancer, and neurodegenerative disorders (Bunsong et al., 2026). According to the World Health Organization, NCDs account for approximately 70% of global mortality, underscoring the importance of dietary strategies aimed at enhancing antioxidant intake (Pisoschi & Pop, 2015; World Health Organization, 2022). Consequently, increasing the consumption of antioxidant-rich foods represents an effective approach to improving systemic antioxidant status and continues to drive interest in food science research and functional food development (Shahidi & Ambigaipalan, 2015).

Mulberry leaves (*Morus* spp.) are a rich natural source of antioxidants, with phenolic compounds particularly flavonoids and phenolic acids being the primary contributors to their antioxidant activity (Pothinuch & Tongchitpakdee, 2019). Owing to these health-promoting properties, mulberry leaves are widely processed into herbal tea products, especially in Asian countries including Thailand. However, during mulberry leaf tea production, some leaves fail to meet commercial quality standards due to undesirable physical characteristics and are classified as low-grade mulberry leaf tea (LGMLT), despite still retaining considerable amounts of phenolic compounds and antioxidant activity. Therefore, LGMLT represents a promising underutilized bioresource for sustainable value-added applications and functional ingredient development. Utilizing LGMLT helps reduce food waste and supports sustainable food processing, while creating value-added antioxidant ingredients for functional food products. Nevertheless, effective application of phenolic-rich residues requires appropriate processing and approaches to improve antioxidant stability and controlled release, highlighting the importance of encapsulation systems (Grgić et al., 2020).

Encapsulation involves entrapping active compounds within a protective polymeric matrix to improve stability and regulate their release behavior (Noipan & Wutticharoenmongkol, 2026). Hydrogels are promising encapsulation systems characterized by

three-dimensional polymeric networks, high water-holding capacity, and the ability to protect bioactive compounds under mild conditions (Grgić et al., 2020). Previous studies have reported that they enhance the stability of grape seed phenolics from winemaking byproducts (Pedrali et al., 2020). These characteristics make hydrogel-based encapsulation a suitable, food-grade platform for antioxidant delivery in functional food applications. Building on these advantages, alginate–protein composite hydrogels have gained increasing interest owing to their tunable structure, scalability, and compatibility with food systems. Alginate was selected as the primary matrix because of its food-grade status and ability to form stable gels via calcium-mediated cross-linking (Draget et al., 2005; Lee & Mooney, 2012). To enhance encapsulation efficiency, protein was incorporated into the hydrogel formulation due to its ability to interact with phenolic compounds through hydrogen bonding and hydrophobic interactions. Protein–polysaccharide systems, particularly alginate–protein hydrogels, have been reported to improve phenolic retention and encapsulation stability through strong intermolecular interactions within the hydrogel matrix (Guo et al., 2018). Whey and soy proteins are commonly used in encapsulation systems because of their functional and gel-forming properties. However, whey protein is animal-derived and associated with dairy allergen concerns, while soy protein may exhibit allergenicity and undesirable flavor characteristics in some applications. In contrast, pea protein has gained increasing attention as a sustainable plant-based protein due to its lower allergenic potential (Boye et al., 2010), compatibility with polysaccharides such as alginate, and suitability for vegan-friendly food formulations. Previous studies have also demonstrated that pea protein can contribute to improved hydrogel network stability and encapsulation performance in bioactive delivery systems (Ali et al., 2026; Thammawong et al., 2025).

Despite growing interest in hydrogel-based encapsulation systems and mulberry-derived antioxidants (Pothinuch & Tongchitpakdee, 2011, 2019; Tchabo et al., 2022; Campos et al., 2025), studies specifically focusing on the integrated extraction, drying and encapsulation of antioxidants of LGMLT remain limited. Therefore, this study developed alginate–pea protein hydrogel beads for encapsulating LGMLT-derived antioxidants and evaluated their physicochemical characteristics, antioxidant activity, and pH-dependent release behavior under acidic and neutral conditions. The results contribute to the development of sustainable,

plant-based encapsulation platforms that support food security by promoting the valorization of tea-processing by-products as value-added food ingredients.

2. Objectives

This study aimed to develop alginate–pea protein hydrogel beads incorporating LGMLT extracts. The effects of extraction and drying methods on the quality and antioxidant properties of LGMLT extracts were investigated. In addition, the effects of different alginate-to-extract ratios on the physicochemical properties, antioxidant activity, and pH-dependent release behavior of the hydrogel beads under acidic and neutral conditions were also evaluated.

3. Materials and Methods

3.1 Materials

LGMLT was obtained from Nong Ya Sai District, Suphan Buri, Thailand. Pea protein was supplied by Abbra Corporation Limited (Thailand). Food-grade sodium alginate (INS 491) and calcium chloride were purchased from Krungthepchemi Company (Thailand). Gallic acid and Trolox were used as standards for total phenolic content and antioxidant assays, respectively. Folin–Ciocalteu reagent and other analytical-grade chemicals were obtained from commercial suppliers. Deionized water was used throughout the experiments.

3.2 Effect of Extraction and Drying Methods on the Quality of LGMLT Extract Powder

For extract powder preparation, 100 g of LGMLT was ground and passed through a 180-mesh stainless steel sieve. The resulting extract powder was mixed with 1,000 mL of deionized water and extracted using decoction and ultrasonication methods.

For decoction, the LGMLT powder was added to deionized water and heated on a hot plate (Model: Morat M22/1 Magnetic Stirrer, Framo Geratetechnik, Germany) at 100°C and soaked for 10 minutes. The mixture was then cooled to room temperature ($27 \pm 2^\circ\text{C}$) and centrifuged at 5,000 rpm and 4°C for 30 min using a refrigerated centrifuge (Model: CR3i, Jouan, France). The supernatant was filtered and the obtained LGMLT extract was subjected to further drying. For ultrasonication extraction, the LGMLT powder–water mixture was sonicated using an ultrasonicator (Cavitator, Mettler Electronics, USA) for 15 min while immersed in an ice bath to maintain the extraction temperature at $4 \pm 1^\circ\text{C}$. Subsequent

centrifugation and filtration were performed as described for the decoction method.

LGMLT extracts obtained from both methods were dried using spray drying and freeze drying. For spray drying, the extract was mixed with 5% (w/v) maltodextrin as a carrier and dried using a spray dryer (LPG 5, FNB Machinery & Solutions, China) at inlet and outlet temperatures of 160 and 90°C, respectively. For freeze drying, the filtered extract was pre-frozen and subsequently lyophilized using a freeze dryer (FreeZone 2.5 Plus, Labconco, USA) under a vacuum pressure of approximately 0.010 mbar. The resulting powders from both drying methods were collected, weighed using a 4-decimal analytical balance (PX224, OHAUS, USA), and the extraction yield (%) was calculated using Eq. (1) (Haideri et al., 2024). The LGMLT extract powder was stored at -18°C for further quality analysis.

$$\text{Extraction yield (\%)} = \frac{\text{Weight of dry extract (g)}}{\text{Weight of raw material (g)}} \times 100 \quad (1)$$

3.3 Effect of Sodium Alginate to LGMLT Extract Ratio on Hydrogel Qualities

Alginate pea protein hydrogels containing LGMLT extract powder were prepared at sodium alginate-to-extract ratios of 2:1, 3:4, and 1:2 (w/w). The ratios were selected through preliminary experiments based on their ability to form stable hydrogel beads with a uniform spherical morphology. The total volume of water was divided into two equal portions. LGMLT powder was dissolved in the first portion of water, followed by the gradual addition of sodium alginate under continuous stirring until a uniform dispersion was obtained. Pea protein (1.3% w/w) was dissolved separately in the second portion of water. The two solutions were combined and homogenized using a hand-held mixer, then allowed to stand at room temperature for 2–3 hours to remove entrapped air. Gelation was achieved by extruding the hydrogel precursor dropwise from a 20 mL syringe into a 1% (w/v) CaCl_2 solution to induce ionic cross-linking. The formed beads were cured for 30 min, drained, rinsed twice with water, and subjected to further quality evaluation. The size of the hydrogel beads was measured using a digital vernier caliper (Series 500, Mitutoyo, Japan).

3.4 Quality Analysis

3.4.1 Encapsulation Efficiency (EE)

Overall encapsulation efficiency (EE) was evaluated using an indirect method based on the

determination of non-encapsulated phenolic compounds remaining in the supernatant after centrifugation, following the method described by Guo et al. (2018) with modification. The hydrogel beads were cured at 4°C for 2 hours to reach equilibrium. After curing, the beads were collected using vacuum filtration. The bead suspension was centrifuged at 5,000 rpm for 15 min at 4°C. Total phenolic content (TPC) in the supernatant was then determined using the Folin–Ciocalteu method. EE was calculated from the difference between the initial TPC incorporated into the hydrogel formulation and the free phenolic content detected in the supernatant using Eq. (2):

$$\text{Overall encapsulation efficiency (\%)} = \frac{m_{\text{initial TPC}} - m_{\text{TPC in curing bath}}}{m_{\text{initial TPC}}} \times 100 \quad (2)$$

where $m_{\text{initial TPC}}$ represents the TPC initially incorporated into the hydrogel formulation, and $m_{\text{TPC in curing bath}}$ represents non-encapsulated phenolic content detected in the supernatant. TPC was determined following section 3.4.3. All measurements were performed in triplicate.

3.4.2 Water Holding Capacity (WHC)

WHC of hydrogel beads was determined following Li et al. (2025) with minor modification. Samples were equilibrated at 4°C for 15 hours, 30 g of hydrogels were centrifuged at 5,000 rpm for 10 min to remove unbound water, and weighed. WHC was calculated using Eq. (3):

$$\text{WHC (\%)} = \left(\frac{M_1}{M_0} \right) \times 100 \quad (3)$$

where M_1 and M_0 are the weights of the hydrogel beads before and after centrifugation (g), respectively. All measurements were performed in triplicate.

3.4.3 Total Phenolic Content (TPC)

TPC was determined using the Folin–Ciocalteu method following Pothinuch et al. (2024) with minor modifications. Sample powder (0.625 g) was extracted with 25 mL of water, and an aliquot (0.1 mL) of the extract was mixed with 0.9 mL water, followed by the addition of 0.1 mL Folin–Ciocalteu reagent and 7% sodium carbonate (1 mL). After incubation for 6 min at room temperature, absorbance

was measured at 760 nm. Gallic acid was used as standard, and results were expressed as mg gallic acid equivalent (GAE)/g. For hydrogel, the samples were freeze-dried and ground prior to analysis. All measurements were performed in triplicate.

3.4.4 Antioxidant Capacity

Antioxidant capacity was evaluated using the ferric reducing antioxidant power (FRAP) assay and the ABTS radical-scavenging assay. Hydrogel samples were freeze-dried, ground, and sieved prior to analysis. The FRAP assay was performed according to Dudonné et al. (2009) with minor modifications. The FRAP reagent, consisting of acetate buffer (pH 3.6), TPTZ, and ferric chloride, was freshly prepared. Sample extract (40 µL) was mixed with 0.2 mL of water and 1.8 mL of FRAP reagent and incubated at 37°C for 30 min, after which the absorbance was measured at 593 nm. The ABTS assay was conducted following Pakamwong et al. (2024) with minor modifications. The ABTS^{•+} radical solution was generated by reacting ABTS with potassium persulfate in the dark for 14–16 hours and diluted to an absorbance of 0.7–0.8 at 734 nm. The ABTS^{•+} solution (2.9 mL) was mixed with the extract (0.1 mL) and incubated for 20 min in the dark before measuring absorbance at 734 nm. Trolox was used as the standard for both antioxidant assays and the results were expressed as mg TE/g. All measurements were performed in triplicate.

3.5 In vitro Releasing Study of LGMLT Extract from Hydrogel

The pH-responsive release profile of bioactive compounds from LGMLT extract hydrogel beads was evaluated following the method of Yoo et al. (2006) with slight modifications. Hydrogel beads (26 g) were immersed in 250 mL of buffer solution adjusted to pH 1.2 and 7.4 to investigate pH-dependent release behavior. The pH 1.2 and 7.4 were selected to represent simulated gastric (USP SGF) and intestinal (USP SIF) conditions, respectively, which represent the physiological pH extremes encountered during oral ingestion. No digestive enzyme was included in the release media. The samples were incubated at 37°C with gentle agitation. Aliquots were collected at 30 min interval over a period of 5 hours. The release media were analyzed for TPC and antioxidant capacities (FRAP and ABTS assays). All experiments were performed in triplicate.

Table 1 Extraction yield (%), total phenolic contents (TPC) and antioxidant capacities (ABTS and FRAP) of low-grade mulberry leaf tea extracts powder obtained by different extraction and drying methods

Extraction	Drying	Extraction yield (%)	TPC (mg GAE/g)	ABTS (mg TE/g)	FRAP (mg TE/g)
Decoction	Spray drying	37.5 ± 0.01 ^b	5.59 ± 0.37 ^b	12.29 ± 0.38 ^c	9.82 ± 0.21 ^b
	Freeze drying	15.8 ± 0.01 ^d	25.41 ± 2.67 ^a	28.33 ± 0.39 ^b	40.41 ± 3.45 ^a
Ultrasonication	Spray drying	39.7 ± 0.01 ^a	6.78 ± 0.23 ^b	13.97 ± 0.40 ^c	11.39 ± 0.20 ^b
	Freeze drying	24.5 ± 0.01 ^c	22.97 ± 2.06 ^a	37.12 ± 0.71 ^a	43.90 ± 5.44 ^a

Note: Different superscript letters (a, b, c and d) indicate statistically significant differences at $p < 0.05$

3.6 Statistical Analysis

The effects of extraction and drying methods on LGMLT extract qualities were studied using a factorial arrangement in a completely randomized design (CRD) as experimental design. Data were analyzed by analysis of variance (ANOVA), and differences among means were compared using Fisher's least significant difference (LSD) test at a significance level of $p < 0.05$. The effects of alginate-to-LGMLT extract powder ratio on the hydrogel characteristics and their pH-responsive release behavior were analyzed using an independent samples t-test to compare the two formulations (2:1 and 3:4) at a significance level of $p < 0.05$. Results were expressed as mean ± standard deviation.

4. Results and Discussion

This study investigated the effects of extraction (decoction and ultrasonication-assisted) and drying (spray drying and freeze drying) methods on the quality of LGMLT extract powder. Antioxidant properties were evaluated through TPC and antioxidant capacity using ABTS and FRAP assays. Extract powder obtained under the optimal processing condition was subsequently used to prepare hydrogel-based encapsulation systems. The effects of alginate-to-LGMLT extract ratio on hydrogel characteristics were examined. The release behavior of encapsulated bioactive compounds was also evaluated under acidic and neutral buffer conditions (pH 1.2 and 7.4) for 5 hours to investigate pH-dependent release behavior of the hydrogel system.

4.1 Physicochemical and Antioxidant Properties of LGMLT Extract Powder

The extraction yield of LGMLT extract powder was in the range of 15.8-39.7%. Similar results have been reported in previous studies. Haideri et al. (2024) reported an extraction yield of approximately 20.12% for phenolic extraction under optimized extraction conditions, while Meesat et al. (2025) reported extraction yields of up to 35.94% for *Centella asiatica*

extracts depending on the extraction method and solvent system used. In this study, both extraction and drying methods significantly affected extraction yield ($p < 0.05$), with a significant interaction observed between the two factors. The highest extraction yield was obtained from ultrasonication-assisted extraction followed by spray drying (Table 1). Spray drying consistently provided higher powder recovery than freeze drying. Although maltodextrin addition generally enhanced powder recovery, the difference between ultrasonication and decoction remained evident within the same drying method, indicating that extraction primarily governed the availability of soluble solids prior to drying, whereas the drying technique mainly influenced solid retention. Ultrasonication enhanced the release of intracellular compounds through cavitation-induced cell disruption, thereby increasing mass transfer and soluble solid concentration in the extract (Chemat et al., 2017; Vilku et al., 2008). Consequently, more material was available for powder formation. In addition, spray drying minimized material loss by reducing stickiness and wall deposition through carrier addition, resulting in improved powder recovery (Tonon et al., 2008; Sidlagatta et al., 2020). In contrast, freeze drying may cause solute migration during freezing and sublimation, leading to partial material loss and lower powder yield (Ratti, 2001). Therefore, the superior yield observed in the ultrasonication-spray drying treatment can be attributed to the combined effects of enhanced soluble solid generation during extraction and reduced solid loss during drying. Spray drying produced higher yields than freeze drying for both extraction methods, primarily due to the use of maltodextrin as a drying aid, which enhanced moisture removal and powder formation. In addition, ultrasonication-assisted extraction promoted the release of bioactive compounds through cavitation-induced cell wall disruption, thereby improving mass transfer and extraction efficiency (Chemat et al., 2017).

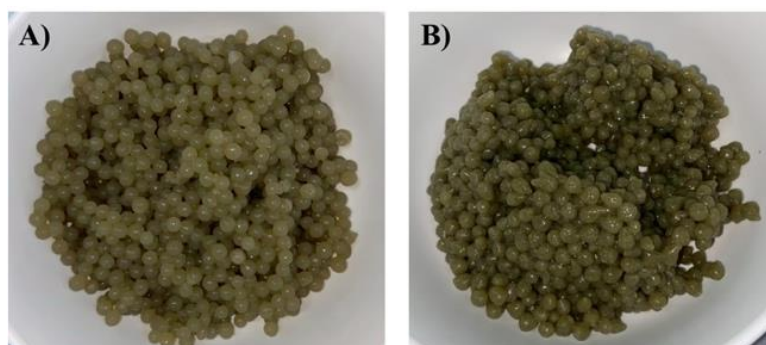


Figure 1 Appearance of low-grade mulberry leaf tea extracts-loaded alginate–pea protein hydrogel beads at sodium alginate-to-mulberry leaf tea extract powder ratios of 2:1 (A) and 3:4 (B)

The effects of extraction and drying methods on the antioxidant properties of LGMLT powder were evaluated based on TPC and antioxidant capacity determined by ABTS and FRAP assays (Table 1). Both factors significantly influenced TPC and antioxidant capacities ($p < 0.05$). Freeze-dried samples consistently exhibited higher TPC and antioxidant capacities than spray-dried samples, regardless of extraction method. This is attributed to the low processing temperature, limited oxygen exposure, and the absence of carrier dilution during freeze drying, whereas spray drying may promote thermal and oxidative degradation of phenolic compounds and reduce apparent bioactive concentration due to maltodextrin addition (Ratti, 2001; Tonon et al., 2008).

The drying method markedly influenced the retention of phenolic compounds and antioxidant capacity. Freeze-dried extracts exhibited significantly higher TPC, ABTS, and FRAP values than their spray-dried counterparts, regardless of the extraction method. Among the freeze-dried samples, decoction and ultrasonication resulted in comparable TPC and FRAP values, with no significant differences observed between the two extraction methods. However, the ultrasonicated freeze-dried extract exhibited significantly higher ABTS activity than the decoction freeze-dried extract. The higher ABTS activity of the ultrasonicated freeze-dried extract, despite its comparable TPC, suggests that antioxidant capacity may depend not only on the total phenolic concentration but also on the phenolic profile and the relative abundance of individual phenolic compounds, which can differ in their radical-scavenging capacity (Heo et al., 2007). In addition, non-phenolic antioxidant constituents may also contribute to the overall antioxidant capacity of plant extracts (Pisoschi & Pop, 2015). In this study, total phenolic content

obtained from ultrasonication-assisted extraction combined with freeze drying (22.97 mg GAE/g DW). This value falls within the range commonly reported for phenolic-rich plant materials, which are generally regarded as promising sources of natural antioxidants when total phenolic content exceeds approximately 15 mg GAE/g (Li et al., 2007). The combination of ultrasonication and freeze drying most effectively preserved antioxidant quality in mulberry leaf residue extract powder. Accordingly, this condition was selected for preparing the extract powder for subsequent hydrogel encapsulation experiments.

4.2 Characteristics of LGMLT Extract Loaded Alginate–Pea Protein Hydrogel Beads

Hydrogel beads loaded with LGMLT extract were prepared using different alginate-to-extract ratios (2:1, 3:4 and 1:2) to evaluate their physicochemical properties and antioxidant capacities. The appearance of the beads is shown in Figure 1. The characteristics of LGMLT extract-loaded alginate–pea protein hydrogel beads, including morphology, encapsulation efficiency, and water holding capacity, were strongly influenced by the alginate-to-extract ratio. Beads prepared at a ratio of 2:1 exhibited a spherical shape and uniform morphology, indicating the formation of a stable and cohesive gel network. In contrast, beads produced at a ratio of 3:4 were less spherical and frequently displayed a teardrop-like morphology, suggesting reduced structural stability during gelation. This behavior may be attributed to the lower relative alginate content, which limited the availability of polymer chains required for effective calcium-mediated ionic cross-linking. At the lowest alginate proportion (1:2 ratio), discrete and well-defined spherical beads were not successfully formed. Instead, the resulting structures were fragile and easily ruptured during handling, indicating insufficient gel

network formation. This instability may be associated with the excessive extract content, which introduced a high concentration of suspended solids and phenolic compounds that interfered with network organization. Polyphenolic compounds can interact with proteins and polysaccharides, forming ternary complexes that disrupt intermolecular interactions necessary for gel stabilization (Jakobek, 2015; Guo et al., 2018; Ali et al., 2026). In addition, phenolics may compete with alginate for calcium ions or alter the spatial arrangement of polymer chains, thereby reducing the formation of the characteristic “egg-box” structure essential for alginate gelation (Draget et al., 2005).

The 2:1 formulation provided a more balanced composition, allowing sufficient alginate chains to participate in ionic cross-linking while maintaining favorable interactions with pea protein. Protein–polysaccharide interactions can promote the formation of an interconnected microgel network, contributing to enhanced structural cohesion and mechanical stability (Le et al., 2017; Zhang et al., 2025). These findings demonstrate that hydrogel bead morphology and structural stability were governed by the balance between polymer availability and extract content. Adequate alginate concentration is essential to establish a continuous and stable gel network, whereas excessive extract loading can disrupt polymer interactions and compromise bead integrity.

The effect of the alginate-to-mulberry leaf residue extract powder ratio on hydrogel bead size is presented in Table 2. Bead size was evaluated only for the 2:1 and 3:4 formulations because the 1:2 formulation did not produce structurally stable beads suitable for measurement. The alginate-to-extract ratio significantly affected bead size ($p < 0.05$), with the 3:4 formulation producing larger beads than the 2:1 formulation. This difference is consistent with the morphological observations discussed previously, in which beads formed at the 3:4 ratio exhibited irregular shapes, including teardrop-like structures and tail-like extensions. These structural irregularities increased the apparent bead size and indicated reduced structural stability during gelation. The lower alginate content in this formulation likely limited the availability of polymer chains for immediate ionic cross-linking upon contact with calcium ions. The droplets were less effectively stabilized and could elongate before complete gel formation, resulting in asymmetric and larger beads. The higher alginate concentration in the 2:1 formulation facilitated rapid

calcium-mediated cross-linking, enabling the droplets to maintain a spherical shape and form a more compact gel network. This rapid stabilization limited droplet deformation and produced smaller and more uniform hydrogel beads (Draget et al., 2005). These findings further demonstrate that alginate concentration governs hydrogel formation, thereby affecting bead morphology and final particle size. This structural difference may also influence encapsulation efficiency and release behavior because bead size and network density affect the diffusion of encapsulated compounds.

The encapsulation efficiency of LGMLT extract-loaded alginate–pea protein hydrogel beads prepared at different alginate-to-extract ratios is presented in Table 2. The alginate-to-extract ratio significantly affected encapsulation efficiency ($p < 0.05$). Beads formulated at a 2:1 ratio exhibited significantly higher encapsulation efficiency than those prepared at a 3:4 ratio. This difference can be attributed to the role of alginate in hydrogel network formation through calcium-mediated ionic cross-linking, commonly described by the egg-box model. A higher alginate content promotes the formation of a more compact and stable gel network, which enhances bead integrity and improves the retention of encapsulated bioactive compounds (Draget et al., 2005). A lower alginate proportion may result in incomplete or less dense gelation, leading to a looser hydrogel structure with reduced ability to retain the extract and, consequently, lower encapsulation efficiency (Lee & Mooney, 2012). In addition to EE, the alginate-to-extract ratio significantly influenced the WHC of the hydrogel beads ($p < 0.05$), as shown in Table 2. WHC was evaluated because it reflects the ability of the hydrogel matrix to retain water, which is associated with network integrity, encapsulation stability, and controlled release behavior. Beads prepared at the 2:1 ratio exhibited a significantly higher WHC than those produced at the 3:4 ratio. Higher WHC values suggest a more stable hydrogel structure with improved water retention, which may enhance the retention of encapsulated phenolic compounds and reduce premature leakage during storage and release. This finding is consistent with the morphological and structural observations discussed previously because the denser, more cohesive gel network formed at higher alginate content can entrap greater amounts of water within the polymer matrix.

Table 2 Effect of alginate-to-extract ratio on particle size, encapsulation efficiency and water holding capacity of LGMLT extract loaded alginate–pea protein hydrogel beads

Alginate : LGMLT ratio	Particle size (mm.)	Overall encapsulation efficiency (%)	Water holding capacity (%)
2:1	3.87 ± 0.09 ^b	95.90 ± 0.24 ^a	55.94 ± 0.80 ^a
3:4	4.56 ± 0.04 ^a	89.49 ± 5.42 ^b	45.02 ± 2.53 ^b

Note: Different superscript letters (a and b) indicate statistically significant differences at $p < 0.05$

Table 3 Effect of alginate-to-extract ratio on Total phenolic content (TPC) and antioxidant capacities (ABTS and FRAP) of LGMLT extract loaded alginate–pea protein hydrogel beads

Alginate : LGMLT Extract Ratio	TPC (mg GAE/g)	ABTS (mg TE/g)	FRAP (mg TE/g)
2:1	2.34 ± 0.12 ^b	1.98 ± 0.25 ^b	1.50 ± 0.18 ^b
3:4	3.61 ± 0.23 ^a	4.39 ± 0.23 ^a	3.04 ± 0.20 ^a

Note: Different superscript letters (a and b) indicate statistically significant differences at $p < 0.05$

Controlling the alginate-to-extract ratio is critical for optimizing both EE and WHC, which are key quality attributes of hydrogel beads intended for the incorporation of bioactive compounds.

The effect of the alginate-to-mulberry leaf residue extract powder ratio on TPC and antioxidant capacity, determined by ABTS and FRAP assays, of alginate–pea protein hydrogel beads is presented in Table 3. The results demonstrated that the formulation ratio significantly influenced the TPC of the hydrogels ($p < 0.05$). Beads prepared at a 3:4 ratio, which contained a higher proportion of extract powder, exhibited significantly higher TPC values than those prepared at a 2:1 ratio. This effect is primarily attributed to the increased extract loading, as LGMLT extract serves as the direct source of phenolic compounds. A higher extract proportion increases the incorporation of phenolics into the hydrogel matrix, resulting in higher measurable TPC values. In contrast, beads formulated with a higher alginate content, although structurally more stable, may form a denser polymer network that restricts the diffusion and extractability of phenolic compounds during analysis, leading to lower observed TPC values (Machado et al., 2022). Moreover, excessively compact gel networks may retain phenolic compounds within the matrix, preventing their complete extraction during the Folin–Ciocalteu assay.

Consistent with the TPC results, the antioxidant capacity of alginate–pea protein hydrogel beads, as evaluated by the ABTS and FRAP assays, was also significantly affected by the alginate-to-extract ratio ($p < 0.05$). Beads prepared at the 3:4 ratio exhibited significantly higher antioxidant activity than those prepared at the 2:1 ratio. This trend reflects

the higher phenolic content in extract-rich formulations, as phenolic compounds are effective antioxidants capable of donating electrons or hydrogen atoms to neutralize free radicals. Conversely, hydrogels with higher alginate content may limit the release of entrapped phenolic compounds during antioxidant assays due to the formation of a denser gel network, resulting in lower measured antioxidant activity (Vallejo-Castillo et al., 2020). Overall, these findings suggest that alginate-to-extract ratio influenced both hydrogel characteristics and antioxidant responses, where higher alginate content improved bead formation, whereas higher extract loading increases phenolic availability and antioxidant activity. Among the tested formulations, the 2:1 ratio demonstrated better structural stability and encapsulation performance, whereas the 3:4 formulation exhibited greater antioxidant release at later stages. Both the 2:1 and 3:4 formulations were selected for further evaluation of pH-responsive release behavior.

4.3 In vitro Releasing Behavior of LGMLT Extract from Hydrogel Beads

The effect of the alginate-to-LGMLT extract powder ratio on the release behavior of alginate–pea protein hydrogel beads was evaluated under acidic (pH 1.2) and neutral (pH 7.4) conditions over a 5 hours period (Figure 2). The release experiment was designed as a preliminary evaluation of the pH-responsive release behavior and stability of phenolic compounds under acidic (pH 1.2) and neutral (pH 7.4) conditions. The effects of different alginate-to-extract ratios on release behaviors were compared within each pH condition.

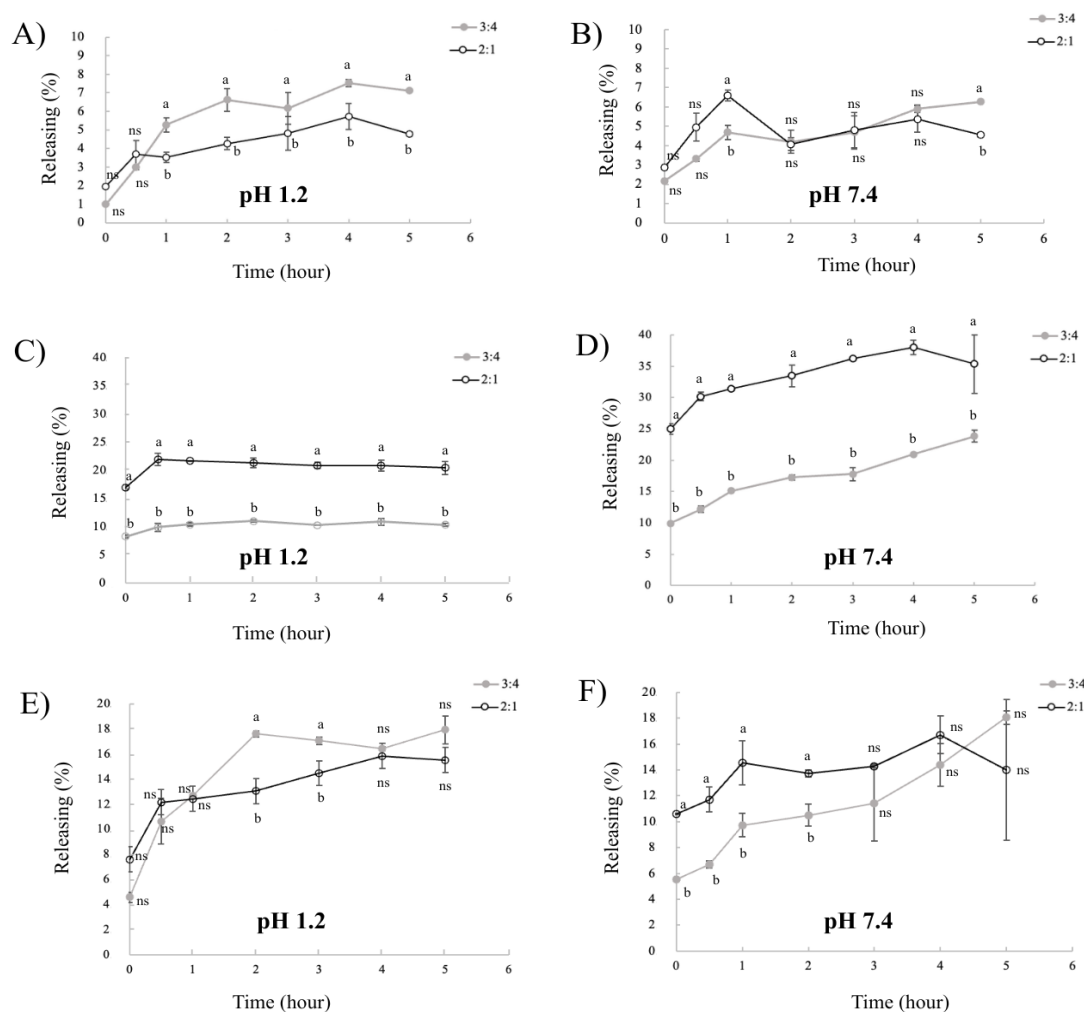


Figure 2 pH-responsive release behavior of total phenolic content (A-B) antioxidant activity determined by the ABTS (C-D), FRAP (E-F) assays from LGMLT extract-loaded alginate-pea protein hydrogel beads under acidic conditions (pH 1.2) and neutral conditions (pH 7.4). Different superscript letters (a and b) indicate significant differences between the 2:1 and 3:4 formulations at the same sampling time ($p < 0.05$); whereas ns indicates no significant difference between formulations

Consistent with the antioxidant characteristics of the hydrogel beads prior to release, the release behavior under acidic conditions (pH 1.2) was formulation-dependent and assay-sensitive. The 3:4 formulation exhibited higher TPC and FRAP activity, whereas the 2:1 formulation showed superior ABTS radical-scavenging capacity. The 3:4 hydrogels consistently released higher TPC and exhibited greater FRAP activity throughout most of the acidic phase ($p < 0.05$), suggesting that the lower alginate content may be associated with faster phenolic diffusion, possibly due to a less dense hydrogel network (Figure 2A and 2E). Similarly, grape pomace phenolics showed faster release from sodium alginate

microbeads under low pH conditions, supporting the role of network stability and protonation in governing release kinetics (Martinović et al., 2023). In contrast, ABTS activity was higher in the release medium from the 2:1 formulation ($p < 0.05$), suggesting that radical-scavenging performance under acidic conditions was governed not only by the quantity of released phenolics but also by their chemical form, accessibility, and interactions within the alginate-pea protein matrix (Figure 2C). These results suggested that variations in gel network structure and protein-phenolic interactions may influence release behavior as well as antioxidant responses measured by different assays.

Under neutral conditions (pH 7.4), the release profiles differed from those observed under acidic conditions, possibly because of alginate deprotonation and changes in hydrogel swelling and permeability. The 2:1 formulation exhibited relatively higher antioxidant activity at the early stage, whereas the 3:4 formulation showed higher TPC and FRAP values at later stages ($p < 0.05$), possibly due to differences in extract loading and hydrogel structure (Figure 2B and 2F). As shown in Figure 2D, ABTS activity remained higher for the 2:1 hydrogel at most time points ($p < 0.05$), whereas FRAP responses became more comparable at later stages. These differences suggest assay-dependent antioxidant responses because each assay evaluates a different antioxidant mechanism. Thus, the variation between assays likely reflects differences in phenolic reactivity after release from the hydrogel matrix rather than changes in antioxidant quantity. However, at pH 7.4, the relatively large error bars observed at 300 min may be attributable to hydrogel swelling (Figure 2D and 2F). Under neutral conditions, deprotonation of alginate carboxyl groups promotes matrix swelling (Wang et al., 2025; Liu et al., 2025), and individual beads may swell to different extents, resulting in variations in pore structure and diffusion behavior (Călina et al., 2024). Because the network mesh size governs solute diffusion (Peppas & Khare, 1993), such non-uniform swelling can lead to heterogeneous diffusion and non-uniform release of encapsulated compounds, thereby increasing variability among replicates. This swelling reflects the intended pH-responsive behavior of the matrix rather than a structural failure. However, the associated bead-to-bead variability represents an experimental limitation that could be reduced in future work by narrowing the bead size distribution or applying a secondary coating. Furthermore, differences in antioxidant responses under neutral conditions may reflect changes in the physicochemical environment of the hydrogel matrix caused by alginate deprotonation and swelling, which could influence protein–phenolic interactions (Jakobek, 2015; Peppas & Khare, 1993).

A comparison of acidic (pH 1.2) and neutral conditions (pH 7.4) showed that, after 300 min, TPC and FRAP release differed only slightly between the two pH conditions, with differences ranging from 0.6% to 12.5%, depending on the formulation. In contrast, ABTS capacity at pH 7.4 was 72.7% and 128.8% higher than that at pH 1.2 for the 2:1 and 3:4 formulations, respectively. The pH-dependent differences were more clearly detected by ABTS than

by TPC and FRAP in this study, which may be attributed to the different antioxidant mechanisms measured by each assay. TPC reflects total phenolic content, FRAP measures reducing power, and ABTS evaluates radical-scavenging activity, which may be more sensitive to changes in antioxidant responses under different pH conditions (Huang et al., 2005). These results suggest that pH influenced the antioxidant responses of the released fractions more markedly than the overall phenolic release. The observed release behavior may be related to the pH-responsive properties of the alginate–pea protein hydrogel matrix. Under acidic conditions (pH 1.2), protonation of alginate carboxyl groups ($-\text{COOH}$) may reduce electrostatic repulsion within the polymer network, resulting in lower swelling and restricted diffusion of encapsulated compounds (Lee & Mooney, 2012). This effect appeared to be more pronounced in the 2:1 formulation, which had a higher alginate content and exhibited lower TPC and FRAP release than the 3:4 formulation. In contrast, deprotonation of alginate ($-\text{COO}^-$) at pH 7.4 may increase matrix expansion and alter the accessibility of released antioxidants, as reflected by the substantially higher ABTS activity observed under neutral conditions. Although a complete gastrointestinal digestion model was not employed, these findings are consistent with the proposed role of alginate-based hydrogels, which may help retain bioactive compounds under acidic conditions and modulate their availability at higher pH. Such pH-dependent behavior highlights the potential of the alginate–pea protein hydrogel system as a food-grade carrier for phenolic-rich extracts by regulating the availability of encapsulated bioactive compounds under different pH conditions. This functionality may support the development of functional food ingredients containing LGMLT-derived antioxidants while promoting the valorization of underutilized mulberry tea processing by-products.

5. Conclusion

This study showed that processing conditions significantly influenced the quality of LGMLT extract powder and its subsequent performance in hydrogel-based encapsulation systems. Ultrasonication-assisted extraction combined with freeze drying was selected for preparing LGMLT extract powder for hydrogel encapsulation because it provided high phenolic retention and antioxidant capacity, particularly ABTS activity, whereas spray drying offered a higher powder yield but lower TPC and antioxidant capacity.

The alginate-to-extract ratio governed hydrogel bead characteristics, including morphology, encapsulation efficiency, water-holding capacity, and antioxidant responses. Alginate-rich formulations (2:1) improved hydrogel characteristics, whereas extract-rich formulations (3:4) increased phenolic loading and antioxidant activity. Among the tested formulations, the 2:1 ratio was considered the most suitable for stable encapsulation due to its better bead formation, encapsulation efficiency, and water-holding capacity. The hydrogels exhibited pH-dependent behavior, showing rapid release at an early stage under acidic conditions (pH 1.2) and increasing release values over time under neutral conditions (pH 7.4). Understanding this pH-responsive release behavior may support the rational design of food-grade encapsulation systems for modulating antioxidant release and phenolic availability under different pH conditions in food application. Future studies should evaluate release behavior at intermediate pH values (pH 5.0–6.8) representative of the small intestine and proximal colon, and should incorporate digestive enzymes to better simulate in vivo conditions. The simulated digestion conditions may provide further insight into the potential applications of the hydrogel system.

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7. Abbreviations

Abbreviation	Full Term
LGMLT	Low-grade mulberry leaf tea
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

FRAP	Ferric reducing antioxidant power
NCDs	Non-communicable diseases
EE	Encapsulation efficiency
TPC	Total phenolic content
WHC	Water holding capacity
GAE	Gallic acid equivalent
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
CRD	Completely randomized design
ANOVA	Analysis of variance
LSD	Fisher's least significant difference

8. CRediT Statement

Jariyawan Pohkhet: Methodology, Formal Analysis, Investigation, Data Curation, Writing – Original Draft

Kamonnat Charoensri: Methodology, Formal Analysis, Investigation

Jutamane Sornnuwat: Methodology, Formal Analysis, Investigation

Chana Panyanon: Resources, Investigation

Nut Thephuttee: Resources, Investigation

Tarit Apisittiwong: Resources, Investigation

Neeracha Sangpreecha: Resources, Investigation

Janisada Wattanasan: Resources, Investigation

Phacharapan Santudprom: Resources, Investigation

Pitchaya Pothinuch: Conceptualization, Methodology, Resources, Validation, Writing – Original Draft, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition

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