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Cardoon flower extract optimization: effects of formulation and preservation

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Short Communication

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Abstract

The pistils of *Cynara cardunculus* L. flowers have long been used as natural coagulants in Portuguese and Spanish cheesemaking, particularly in protected designation of origin cheeses. Despite its historical relevance, standardized purified formulations are still unavailable, leading producers to rely on artisanal methods that result in variability in extract composition and cheese quality. This study evaluates the effect of extraction formulation and storage conditions on the technological and microbiological properties of cardoon flower extracts. A preliminary trial identified effective NaCl concentrations, guiding the selection of 5% and 20% (w/v) solutions. In the main trial, extracts were prepared using maceration and filtration with four solutions: 5% and 20% (w/v) NaCl, acetate buffer (pH 5.5) and citrate buffer (pH 3.0), stored at 4 °C for 135 days with 2% potassium sorbate. Technological properties and microbial characteristics were monitored throughout a 135-day storage period. Citrate buffer extracts showed higher enzymatic stability and microbial control, while the 5% NaCl formulation was associated with favourable technological performance indicators, including higher curd firmness and consistent milk-clotting activity. These results support the use of 5% NaCl extracts as a promising plant-based coagulant, offering a balanced solution for enzymatic efficiency and preservation in cheese production.

Various plant species, including those from the genera *Cynara, Ficus, Galium* and *Withania*, have traditionally been used as milk coagulants in cheesemaking (Jacob *et al.*, 2011; Amira *et al.*, 2017). Among them, cardoon (*Cynara cardunculus* L.) is a well-established plant-based coagulant in traditional cheesemaking, particularly in Portugal and Spain, where its use is mandatory in several protected designation of origin (PDO) cheeses (Commission Regulation [EC] No. 1107/96 1996; Conceição *et al.*, 2018). The dried pistils, rich in milk-clotting aspartic proteases, impart distinctive sensory attributes and meet the growing demand for vegetarian and minimally processed ingredients (Fernández *et al.*, 2006; Amira *et al.*, 2017; Gostin and Waisundara, 2019). Beyond PDO products, cardoon extracts are increasingly adopted in non-traditional and industrial cheese production (Almeida and Simões, 2018).

Cheeses produced using vegetable coagulants, particularly extracts obtained from *C. cardunculus* L. flowers, differ markedly from cheeses made with animal rennet. These cheeses generally exhibit unique textural and sensory characteristics resulting from the particular proteolytic activity of plant-derived enzymes (Roseiro *et al.*, 2003; Galán *et al.*, 2012; Fox *et al.*, 2017a). These properties result from the higher and less specific proteolytic activity of cardoon enzymes, which affects curd structure, moisture retention, texture and flavour development (Conceição *et al.*, 2018; Alvarenga *et al.*, 2024). However, enzyme extraction from cardoon flowers remains largely artisanal, leading to significant batch variability influenced by factors such as plant ecotype, flower maturity and drying or maceration conditions (Roseiro *et al.*, 2003; Lambré *et al.*, 2022). The lack of standardized formulations, together with seasonal raw material availability, continues to limit broader industrial application (Barracosa *et al.*, 2021).

During extract preparation, NaCl is commonly incorporated to support enzyme maceration and potentially limit microbial growth, yet its use is often empirical (Tsouli, 1974; Conceição *et al.*, 2018). Moreover, traditional aqueous extracts show rapid loss of activity and low microbial stability during refrigerated storage, underscoring the need for improved formulation and preservation strategies. Despite the long history of cardoon use, few studies have systematically

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evaluated how these factors jointly affect enzymatic performance and safety (Tavaria *et al.*, 1997; Conceição *et al.*, 2018; Alavi and Momen, 2020). This study investigates the influence of NaCl concentration and preservation methods on the enzymatic and microbiological characteristics of cardoon flower extracts, aiming to support the development of more stable, standardized and industry-ready plant coagulants.

Materials and methods

Plant material

Cardoon (*C. cardunculus* L.) flowers were collected from an ecotype cultivated at the INIAV campus in Oeiras, Portugal. Only the upper purple portions of the flowers (pistils) were manually separated, cleaned to remove debris and dried at room temperature (20–25°C) for about 30 days in a controlled, shaded environment to preserve enzymatic activity and ensure sample quality.

Preliminary evaluation of NaCl concentration

A preliminary assay was carried out to evaluate how increasing NaCl concentrations affect the enzymatic activity of cardoon flower extracts. Dried pistils (0.04 g/L) were macerated in salt solutions from 0% to 20% (w/v), in 2.5% increments, and all tests were performed in triplicate.

Main trial: preparation of cardoon flower extracts using four different extraction formulations

Based on preliminary results, four extraction solutions were selected: NaCl at 5% (optimal enzymatic performance) and 20% (high-salt condition for potential microbial inhibition), plus two buffer solutions: sodium acetate (pH 5.5), following ISO 23058/IDF 199:2006 (ISO and IDF, 2006), and citrate (pH 3.0), chosen for its acidity and potential antimicrobial effect. Each formulation was prepared in duplicate as independent biological replicates, yielding eight 50 mL extracts.

Dried pistils (2 g) were manually macerated at room temperature with each extraction solution following traditional cheesemaking procedures, then filtered (Whatman No. 40) to a final volume of 50 mL (Gomes *et al.*, 2019, 2024). Extracts were prepared under controlled laboratory conditions, supplemented with 2% potassium sorbate and stored at 4 °C. Enzymatic and microbiological properties were evaluated at 0, 45, 90 and 135 days, with all assays conducted in duplicate.

Evaluation of technological properties

In the preliminary trial, milk-clotting activity (MCA) was determined according to ISO 23058/IDF 199:2006 (ISO and IDF, 2006) and expressed as international milk-clotting units (IMCU/g), reflecting the activity per gram of dried flower material. In the main trial, MCA was expressed as IMCU/mL to quantify enzymatic activity in the liquid extracts.

Coagulation dynamics were assessed using an Optigraph (Alliance, Frépillon, France), which measures near-infrared signal attenuation during micelle aggregation. This allowed real-time determination of parameters such as flocculation time (R) and curd firmness indices (AR, A2R, A20, A40), following the procedures described by Gomes *et al.* (2019, 2024).

Microbiological analysis

Microbiological characterization during the main trial was performed at each storage time under sterile conditions. The following microbial groups were selected due to their relevance to extract spoilage and food safety: (1) Total mesophilic bacteria were enumerated on Plate Count Agar (PCA) (Merck KGaA, Darmstadt, Germany) at 30°C for 72 h (ISO 4833–1:2013/Amd 1:2022). (2) Following ISO 21527-2:2008, moulds and yeasts on Rose Bengal Chloramphenicol Agar (VWR International BV, Leuven, Belgium) at 25°C for 5–7 days. Results for moulds and yeasts are reported separately in Table 3 for clarity. (3) Enterobacteriaceae on Violet Red Bile Glucose Agar (Biokar Diagnostics, Beauvais, France) plates, at 37°C for 24 h (ISO 21528-2:2017). (4) Psychrotrophic microorganisms on PCA at 6.5°C for 10 days (ISO 17410:2019).

Statistical analysis

Statistical analyses were performed using STATISTICA for Windows (version 7.0, StatSoft Inc., Tulsa, OK, USA). One-way analysis of variance followed by the Scheffé post hoc test was applied to evaluate the effect of NaCl concentration in the preliminary trial ($n = 3$), with a 95% confidence level ($P < 0.05$).

Due to the limited number of biological replicates in the main trial ($n = 2$), results were analysed descriptively and are presented as mean values ± standard deviation.

Results and discussion

Preliminary trial with increasing NaCl concentrations

The preliminary assay assessed the effect of increasing NaCl concentrations, revealing that moderate salt levels, particularly 5% NaCl, provided favourable coagulation performance, with higher MCA and stronger curd formation. Intermediate NaCl concentrations improved enzymatic performance relative to the unsalted control, whereas both low and high salt levels were associated with delayed flocculation and weaker curd formation, indicating reduced efficiency (Table 1). These results reinforce earlier findings showing that elevated NaCl levels can inhibit the proteolytic system of *C. cardunculus* L. flowers (Sousa and Malcata, 1996). The 5% NaCl formulation showed a favourable balance between milk-clotting performance and curd structure and is also expected to limit microbial growth, supporting extract stability during storage, in agreement with Conceição *et al.* (2018).

Technological properties of the extracts prepared with four extraction formulations

Table 2 summarizes the technological performance of the extracts obtained with the four formulations. The 20% NaCl extract consistently showed the lowest MCA values across all storage times, suggesting an inhibitory effect of high salt levels. As expected, MCA trends followed those of the flocculation time (R), which increased over storage, particularly in the 20% NaCl formulation (Dias *et al.*, 2025). Initial MCA values were consistent with the preliminary trial after unit conversion, indicating consistency between the preliminary and main trials.

Gel firmness measured at twice the coagulation time (AR) decreased during storage due to increasing R values, with lower firmness at the end of the trial (Fox *et al.*, 2017a, 2017b). The

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Table 1. Mean values (standard deviations) of the technological properties of the cardoon flower extracts with 2.5% increments of NaCl concentrations

	Technological properties					
NaCl concentrations	MCA (IMCU/g)	R (s)	AR (V)	A2R (V)	A20 (V)	A40 (V)
0% (control)	36.02 (17.61)^{b}	1632 (113)^{a}	2.57 (0.23)^{a}	3.95 (0.35)^{a}	0 (0)^{b}	1.62 (0.44)^{ab}
2.5%	59.84 (4.07)^{abc}	1048 (44)^{bc}	2.07 (0.16)^{ab}	3.15 (0.22)^{ab}	0.44 (0.16)^{ab}	2.45 (0.28)^{a}
5%	85.46 (3.77)^{a}	840 (35)^{c}	1.76 (0.22)^{b}	2.66 (0.32)^{b}	0.94 (0.13)^{a}	2.56 (0.30)^{a}
7.5%	62.39 (4.27)^{ab}	1053 (141)^{bc}	2.18 (0.22)^{ab}	3.43 (0.36)^{ab}	0.42 (0.36)^{ab}	2.58 (0.30)^{a}
10%	53.87 (10.95)^{bc}	1127 (153)^{bc}	2.06 (0.20)^{ab}	3.21 (0.28)^{ab}	0.28 (0.25)^{ab}	2.25 (0.44)^{ab}
12.5%	57.22 (8.79)^{bc}	1087 (137)^{bc}	1.99 (0.27)^{ab}	3.06 (0.43)^{ab}	0.31 (0.29)^{ab}	2.26 (0.30)^{ab}
15%	46.74 (14.51)^{bc}	1382 (63)^{ab}	2.36 (0.22)^{ab}	3.71 (0.33)^{ab}	0 (0)^{b}	1.87 (0.03)^{ab}
17.5%	37.97 (0.59)^{bc}	1597 (30)^{a}	2.12 (0.07)^{ab}	3.33 (0.07)^{ab}	0 (0)^{b}	1.25 (0.03)^{b}
20%	39.07 (8.80)^{bc}	1390 (79)^{ab}	2.04 (0.17)^{ab}	3.12 (0.27)^{ab}	0 (0)^{b}	1.64 (0.28)^{ab}

MCA, milk-clotting activity; R, coagulation time; AR and A2R, curd firmness after two and three times the coagulation time (R) after coagulant addition. respectively, A20 and A40, curd firmness at 20 and 40 min, respectively, after coagulant addition; IMCU/g, International Milk-Clotting Units; s, seconds; V, volts. Different superscript letters in each column indicate significant differences ($P < 0.05$, $n = 3$, Scheffé test).

Table 2. Mean values (standard deviations) of the technological properties of the extracts during the storage period

		Extraction formulation			
Technological Properties	Storage period (days)	20% NaCl	5% NaCl	Citrate pH 3.0	Acetate pH 5.5
MCA (IMCU/mL)	0	2.15 (0.07)	3.50 (0.07)	3.20 (0.85)	3.45 (0.78)
	45	1.60 (0.71)	2.90 (0.71)	3.10 (1.27)	3.20 (1.13)
	90	2.05 (0.78)	3.50 (0.42)	4.30 (0.57)	4.25 (0.21)
	135	1.40 (0.00)	3.55 (0.35)	4.00 (0.28)	4.15 (0.64)
R (s)	0	959 (22.58)	837 (33.60)	641 (16.18)	746 (20.17)
	45	1396 (160.87)	919 (8.84)	863 (159.81)	887 (199.06)
	90	1460 (103.95)	861 (112.79)	826 (8.49)	831 (34.65)
	135	1271 (86.27)	716 (11.31)	658 (66,11)	679 (41.01)
AR (V)	0	0.97 (0.11)	1.69 (0.35)	1.08 (0.14)	0.90 (0.06)
	45	1.75 (0.46)	1.58 (0.66)	1.23 (0.13)	1.44 (0.52)
	90	1.48 (0.01)	2.07 (0.03)	1.39 (0.05)	1.16 (0.09)
	135	1.34 (0.07)	1.97 (0.03)	1.38 (0.02)	1.25 (0.00)
A2R (V)	0	1.60 (0.10)	2.73 (0.67)	1.75 (0.25)	1.47 (0.09)
	45	2.83 (0.81)	2.56 (1.11)	2.05 (0.21)	2.32 (0.80)
	90	2.39 (0.03)	3.30 (0.06)	2.26 (0.08)	1.85 (0.11)
	135	2.19 (0.08)	3.09 (0.10)	2.16 (0.01)	1.94 (0.02)
A20 (V)	0	0.30 (0.08)	0.80 (0.24)	0.97 (0.14)	0.58 (0.09)
	45	0.00 (0.00)	0.58 (0.16)	0.59 (0.36)	0.59 (0.14)
	90	0.00 (0.00)	0.92 (0.40)	0.68 (0.07)	0.56 (0.02)
	135	0.00 (0.00)	1.44 (0.06)	1.26 (0.12)	1.02 (0.13)
A40 (V)	0	1.27 (0.03)	2.63 (0.64)	2.11 (0.31)	1.56 (0.12)
	45	1.31 (0.05)	2.23 (0.97)	1.93 (0.55)	2.04 (0.25)
	90	1.02 (0.13)	3.07 (0.34)	2.19 (0.09)	1.78 (0.04)
	135	1.21 (0.08)	3.37 (0.14)	2.59 (0.09)	2.19 (0.06)

Abbreviations for technological properties follow those defined in Table 1.

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Table 3. Mean values (standard deviations) of microbial counts (log CFU/mL) for cardoon flower extracts during storage (0–135 days), under different extraction formulations

		Extraction formulation			
Microbial groups (log CFU/mL)	Storage period (days)	20% NaCl	5% NaCl	Citrate pH 3.0	Acetate pH 5.5
Total mesophilic bacteria	0	1.50 (0.28)	1.93 (0.19)	0.40 (0.00)	1.56 (0.42)
	45	0.89 (0.49)	1.04 (0.23)	n.d.	0.71 (0.15)
	90	0.93 (0.04)	1.27 (0.38)	n.d.	0.74 (0.00)
	135	1.20 (0.00)	1.15 (0.01)	n.d.	0.54 (0.76)
Moulds	0	0.15 (0.21)	0.39 (0.55)	0.60 (0.85)	1.33 (1.46)
	45	n.d.	0.15 (0.21)	n.d.	0.45 (0.21)
	90	n.d.	n.d.	n.d.	0.15 (0.21)
	135	n.d.	n.d.	n.d.	n.d.
Yeasts	0	n.d.	0.30 (0.43)	1.06 (1.49)	1.33 (1.46)
	45	n.d.	n.d.	n.d.	n.d.
	90	n.d.	n.d.	n.d.	n.d.
	135	n.d.	n.d.	n.d.	n.d.
Enterobacteriacea	0	n.d.	n.d.	n.d.	n.d.
	45	n.d.	n.d.	n.d.	n.d.
	90	n.d.	n.d.	n.d.	n.d.
	135	n.d.	n.d.	n.d.	n.d.
Psychrotrophic microorganisms	0	n.d.	n.d.	n.d.	n.d.
	45	n.d.	n.d.	n.d.	n.d.
	90	n.d.	n.d.	n.d.	n.d.
	135	n.d.	n.d.	n.d.	n.d.

n.d., not detected (below the limit of detection of the method, corresponding to 0 log CFU/mL, according to ISO standards).

5% NaCl extract maintained higher curd firmness at later storage times compared with the other extraction formulations. Overall, storage time did not affect this parameter. A similar pattern was observed for A2R, which reflects structural development beyond initial coagulation and is relevant for firmer cheese types. The 5% NaCl formulation showed higher A2R values at 90 days, although no clear differences were observed across storage times for this parameter.

For the A20 parameter, storage time affected only the 5% NaCl extract, which showed higher firmness at later stages due to its lower R value, allowing curd development within the 20-min interval. Formulation type also influenced A20: at the initial time, the citrate buffer produced the lowest R and highest firmness, while the 20% NaCl extract did not initiate coagulation within 20 min at any storage point, explaining the absence of measurable firmness. The A40 parameter, less dependent on R, did not vary over storage time but showed differences among formulations.

Several extraction systems, including water, saline solutions and buffered systems, have been reported for obtaining milk-clotting enzymes from *C. cardunculus* flowers, with extraction conditions strongly influencing coagulation properties and curd firmness (Ben Amira *et al.*, 2017). In this study, 5% NaCl appeared to be a promising formulation, providing higher MCA and improved gel firmness, while higher salt concentrations reduced activity, consistent with previous reports on the inhibitory effects of excessive NaCl on the proteolytic system of *C. cardunculus* L. enzymes (Sousa and Malcata, 1996; Conceição *et al.*, 2018), which is consistent with the reduced activity observed in the 20% NaCl extracts.

Although the NaCl introduced through cardoon extracts is negligible due to the low dosages used in cheesemaking, extraction conditions may still indirectly influence cheese quality by affecting coagulation kinetics and curd structure, with the 5% NaCl extract showing favourable technological characteristics under the conditions tested (Fox *et al.*, 2017a, 2017b; Alvarenga *et al.*, 2024).

The effect of NaCl on MCA and coagulation dynamics is likely linked to changes in ionic strength affecting the stability and activity of cardoon aspartic proteases. Moderate salt concentrations may support enzyme activity, while higher levels can inhibit proteolysis, explaining the reduced MCA and delayed flocculation observed at 20% NaCl (Tsouli, 1974; Sousa and Malcata, 2002). Similar sensitivity of cardoon proteases to extraction conditions has also been reported in more recent studies (Alavi and Momen, 2020).

The results also show that enzymatic performance and microbial stability depend on the combined effects of pH and ionic environment. Cardoon proteases remain active under moderately acidic conditions, preserving MCA in citrate-based extracts, while citrate and low pH contribute to microbial inhibition (Alavi and Momen, 2020; Coban, 2020; Lambré *et al.*, 2022). Overall, the technological performance and preservation of cardoon flower extracts appear to depend on the interplay between enzyme activity, ionic strength and pH, factors that may ultimately influence cheese texture and quality (Fox *et al.*, 2017a, 2017b; Alvarenga *et al.*, 2024).

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Microbiology evaluation

The citrate buffer was associated with the lowest mesophilic bacterial counts, with no detectable mesophilic bacteria observed during refrigerated storage (Table 3). In contrast, both saline extract formulations maintained detectable mesophilic counts throughout storage, indicating that salt alone was not sufficient to suppress growth under the tested conditions. These results align with Coban (2020), who reported that organic acids exert antimicrobial activity by disrupting membrane integrity and interfering with cellular processes, supporting the enhanced inhibitory effect observed in the citrate formulation.

Fungal growth was detected at the beginning of storage but was absent in all formulations by the end of the trial, with moulds contributing more to early fungal counts than yeasts. Yeasts appeared only at the initial evaluation, and notably, the 20% NaCl extract showed no yeast presence at any time. Enterobacteriaceae and psychrotrophic microorganisms were undetectable across all formulations and storage periods. The addition of 2% potassium sorbate likely contributed to suppressing microbial growth, although the persistent presence of mesophilic bacteria indicates that inhibition was not complete.

Given that cardoon extract is typically used at very low dosages in cheesemaking (0.2–0.3 g/L) (Alvarenga *et al.*, 2021, 2024), the resulting potassium sorbate concentration in cheese would be around 0.1 g/kg, well within regulatory safety limits (Regulation [EC] No. 1332/2008). Overall, microbial loads remained low, though not fully eliminated, contrasting with reports on lyophilized extracts, which show greater stability during storage (Tejada *et al.*, 2008).

In addition to formulation and preservation strategies, the microbiological safety of cardoon flower extracts strongly depends on good hygiene practices throughout flower handling and extract preparation. Proper cleaning of raw plant material, controlled drying conditions, sanitary maceration and filtration procedures and the use of food-grade water and additives are essential to minimize initial microbial contamination. Several authors have emphasized that traditional preparation methods may lead to variable microbial loads if hygienic conditions are not adequately controlled (Tejada *et al.*, 2008; Conceição *et al.*, 2018).

Therefore, although the present study suggests that formulation, particularly the use of citrate buffer or moderate NaCl concentrations, can contribute to improved microbial stability during storage, these strategies should be considered complementary to hygienic processing practices rather than stand-alone safety measures. This integrated approach is particularly relevant when scaling up artisanal cardoon extract production for broader industrial application, as also highlighted in safety assessments of *C. cardunculus* L. enzymes for food use (Lambré *et al.*, 2022).

The use of vegetable coagulants in cheesemaking has traditionally depended on empirical knowledge and artisanal practices, which contribute to the typical texture and sensory identity of these cheeses (Roseiro *et al.*, 2003; Conceição *et al.*, 2018). Thus, the variability associated with plant-based coagulants should be seen not only as a technological challenge but also as an intrinsic feature of traditional cheesemaking.

Although this study focuses on *C. cardunculus* L., the species traditionally used in Iberian PDO cheesemaking, other *Cynara* species, such as *Cynara humilis* L., are also used as vegetable coagulants in Mediterranean regions. Differences in species, ecotype and floral composition are known to influence enzyme profiles, MCA and proteolytic behaviour within *Cynara* spp. (Roseiro *et al.*, 2003; Amira *et al.*, 2017; Alavi and Momen, 2020).

C. cardunculus L. was selected for this study due to its technological relevance, mandatory use in several PDO cheeses and the fact that its enzymes have been evaluated and approved for food use by the European Food Safety Authority (Lambré *et al.*, 2022). Therefore, while the effects of pH and ionic strength observed here may be broadly relevant to plant-based coagulants, caution is required when extrapolating these findings to other *Cynara* species or botanical sources, and further comparative studies are needed to assess their wider applicability.

A limitation of the present study is the lack of validation in actual cheesemaking systems, as all results were obtained under controlled laboratory conditions. In real cheese manufacture, factors such as milk composition, processing parameters and production scale are known to significantly influence coagulation behaviour, curd structure and final cheese quality, particularly when vegetable coagulants are used (Roseiro *et al.*, 2003; Galán *et al.*, 2012; Fox *et al.*, 2017a, 2017b). Therefore, while the proposed extraction strategies show promising effects at the extract level, their practical relevance should be confirmed through pilot or industrial-scale cheesemaking trials (Alvarenga *et al.*, 2024).

In conclusion, the preliminary trial confirmed that cardoon flower extracts prepared with 5% NaCl achieved the highest MCA and the best coagulation performance, while very low and high salt concentrations impaired enzymatic efficiency and delayed curd formation. Across the four extraction formulations tested in the main trial, the 5% NaCl solution consistently delivered the most favourable technological properties, including higher gel firmness and more efficient coagulation dynamics. The citrate buffer also performed well, particularly in terms of MCA, whereas the acetate buffer showed intermediate behaviour. As expected, the 20% NaCl extract exhibited the lowest activity and slowest coagulation, confirming its limited suitability for cheesemaking applications.

These results provide practical guidance for improving the consistency and shelf-life of cardoon flower extracts, supporting their use in both traditional and standardized cheesemaking. Optimizing extraction formulations may reduce variability associated with artisanal preparation while maintaining the properties required for high-quality cheese production. Future studies should validate these strategies at pilot or industrial scale and assess their applicability to other *Cynara* species and plant-based coagulants.

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