

Biochemical and Technofunctional Dynamics of Botanical Milk Coagulants: A Comprehensive Blueprint for Harvesting, Dehydration, and Aqueous Extraction of *Cynara cardunculus* and *Urtica dioica*

1. Introduction to Plant-Derived Dairy Coagulation and Matrix Chemistry

The global dairy industry relies fundamentally on the enzymatic destabilization of the milk matrix, a complex biochemical transformation traditionally mediated by animal rennet—specifically, calf chymosin extracted from the abomasum of unweaned calves¹. Bovine milk functions as a highly stable colloidal suspension where casein proteins, which constitute approximately 80% of the total nitrogen in bovine milk, assemble into highly hydrated, spherical structures known as casein micelles¹. These micelles are composed of four primary fractions:

α_{s1} -, α_{s2} -, β -, and κ -caseins in a general ratio of 40:10:35:12³. The micellar core is stabilized by colloidal calcium phosphate cross-links and hydrophobic interactions among the highly phosphorylated α_{s1} -, α_{s2} -, and β -caseins, while the exterior is maintained by a "hairy" layer of κ -casein¹. The C-terminal region of native κ -casein is distinctly hydrophilic and carries a net negative charge at the native pH of milk (approximately 6.7), providing the micelle with colloidal stability through both steric hindrance and electrostatic repulsion¹.

The primary phase of cheesemaking requires the targeted proteolytic cleavage of the

$Phe_{105} - Met_{106}$ peptide bond of this protective κ -casein layer¹. This highly specific hydrolysis releases the hydrophilic, negatively charged caseinomacropeptide (CMP) into the

soluble whey fraction, leaving the hydrophobic para- κ -casein integrated within the micelle

core¹. Upon the removal of approximately 85% to 90% of the κ -casein barrier, the zeta potential of the micelle drops by roughly 50%, extinguishing the electrostatic repulsion¹. This triggers the secondary, non-enzymatic phase of coagulation, wherein the destabilized micelles cooperatively aggregate via hydrophobic interactions and calcium-mediated ionic cross-links to form a three-dimensional fractal gel network, ultimately expelling liquid whey during syneresis to

form solid curd¹.

Historically, this precise proteolysis was achieved exclusively using animal rennet. However, fluctuating supply chains, the rising cost of animal extraction, strict religious dietary parameters (such as Halal and Kosher restrictions), and surging global demand for vegetarian products have catalyzed a profound paradigm shift toward botanical coagulation agents¹. Additionally, the

rise of Alpha-gal syndrome—a severe, tick-borne allergy to galactose- α -1,3-galactose, a carbohydrate found in all non-primate mammalian tissues—has created a strict medical necessity for authentic, mammalian-free dairy coagulants¹. While microbial coagulants and Fermentation-Produced Chymosin (FPC) currently dominate the industrial market, the artisanal and "clean label" sectors are experiencing a renaissance in the application of native phytoproteases¹. Botanical coagulants are deeply embedded in Mediterranean cheesemaking traditions and remain a mandatory regulatory requirement for the production of several European Protected Designation of Origin (PDO) cheeses, including Serra da Estrela, Torta del Casar, Queso de La Serena, and Azeitao¹.

The transition from ancient ethnobotanical practices—such as brewing simple floral "teas" for curdling—to modern, scalable food biotechnology presents a highly complex matrix of variables. Successful integration of botanical coagulants, specifically those derived from the wild cardoon (*Cynara cardunculus*) and the stinging nettle (*Urtica dioica*), demands exacting precision. The dairy technologist must master harvesting phenology, thermodynamic control during dehydration, electrochemical optimization during aqueous extraction, and stringent parasitological mitigation.

2. The Ecological, Legal, and Toxicological Framework of Foraging

For artisanal cheesemakers operating in peri-urban environments, such as the Berlin-Brandenburg region of Germany, sourcing botanical coagulants through wild foraging requires navigating a stringent legal architecture designed to protect biodiversity. Furthermore, the modern landscape introduces severe toxicological and parasitological risks that demand systematic technological mitigation prior to any cheese production¹.

The extraction of wild flora in Germany is governed federally by the Bundesnaturschutzgesetz (BNatSchG), which strictly prohibits the devastation of wild plants from their natural locations without reasonable cause¹. The law provides an exemption known as the *Handstraußregelung* (hand bouquet rule) under § 39 Abs. 3 BNatSchG, which permits individuals to harvest small quantities of aerial plant parts for immediate personal consumption¹. However, this legal framework explicitly excludes commercial harvesting. A cheesemaker intending to forage *Urtica dioica* or cultivate wild strains of *Cynara cardunculus* for commercial sale must obtain explicit written consent from the landowner and formal collection permits from conservation authorities¹. The unpermitted excavation of subterranean root structures is an absolute prohibition and constitutes property damage under the Strafgesetzbuch (Criminal Code)¹.

Beyond legal restrictions, urban foraging introduces heavy metal bioaccumulation risks.

Ubiquitous ruderal species frequently targeted by foragers, notably *Urtica dioica*, possess highly

adaptable root systems capable of intense phytoextraction of soil contaminants, including lead (Pb), zinc (Zn), copper (Cu), cadmium (Cd), and chromium (Cr)¹. The physiological distribution of these heavy metals is not uniform; cadmium and copper preferentially accumulate in the foliar tissues (leaves) of the nettle, while chromium concentrates heavily in floral structures¹. Because the preparation of botanical coagulants requires the intensive concentration of these exact tissues, foraging near high-traffic arteries or former industrial brownfields introduces a severe risk of heavy metal transfer directly into the concentrated botanical "tea" and, subsequently, the cheese matrix¹.

The paramount biological risk associated with foraging low-growing flora in Central Europe is exposure to *Echinococcus multilocularis*, the fox tapeworm¹. Definitive hosts, primarily red foxes (*Vulpes vulpes*) maintaining extraordinarily high population densities in urban spaces, excrete highly resilient microscopic eggs via their feces¹. Humans act as aberrant intermediate hosts, and the ingestion of these eggs via contaminated plant extracts leads to alveolar echinococcosis—a progressive, tumor-like infiltration of the liver with a remarkably prolonged incubation period of 10 to 15 years, which is highly lethal if left untreated¹.

Standard washing protocols and domestic freezing (-20°C) are entirely ineffective against the highly adhesive and environmentally resilient eggs of *E. multilocularis*, which require sustained temperatures of -80°C for days or heating to 70°C to 80°C for several minutes for definitive destruction¹. This creates an absolute technological paradox for the cheesemaker: subjecting botanical coagulant extracts to 70°C induces catastrophic thermal denaturation of the very aspartic and cysteine proteases required for milk coagulation¹. Because thermal pasteurization of the botanical extract is biochemically impossible, the operator must utilize mechanical microfiltration to resolve the biological hazard. The taeniid eggs of *E. multilocularis* measure

approximately 30 to 35 μm in diameter¹. By preparing the cold aqueous enzyme extract and passing it through a commercial polyethersulfone (PES) membrane filter with a sterilizing pore size of 0.22 μm or 20 μm , the operator guarantees the physical exclusion of the parasite eggs while allowing the soluble, low-molecular-weight proteases (ranging from 14 kDa to 48 kDa) to pass unimpeded into the sterile filtrate¹.

3. Agronomy and Climate-Adaptive Cultivation

To bypass the legal ambiguities, toxicological soil risks, and complex zoonotic threats inherent to urban foraging, the localized agricultural cultivation of coagulant species represents the most secure supply chain strategy for artisanal production¹.

Historically, the perennial cultivation of Mediterranean species such as *Cynara cardunculus* in Northern and Central Europe was severely limited by winter frost events. The United States Department of Agriculture (USDA) hardiness zones classify geographic regions based on average minimum winter temperatures. For decades, regions like Berlin-Brandenburg were firmly situated in Zones 6b to 7a, enduring sustained deep freezes (-20.4°C to -15.0°C) that caused lethal vascular damage to the overwintering rootstocks of artichoke thistles¹.

However, anthropogenic climate change and the localized urban heat island effect have fundamentally shifted European agricultural geography. Updated climatological models

(1991–2020) indicate that Central German regions have shifted decisively into Zone 7b (-14.9°C to -12.3°C) and increasingly into Zone 8a (-12.2°C to -9.5°C), effectively mimicking the historical winter climate profiles of southern France or northern Italy¹. This upward shift in winter minimums makes the perennial cultivation of *C. cardunculus* highly feasible in northern latitudes¹.

C. cardunculus follows an annual growth cycle, germinating in the autumn, overwintering in a rosette stage, and sending up rapidly elongating stalks in the spring to flower in the early summer⁶. It is a robust, deep-rooted crop highly adapted to low-input agriculture¹⁴. It exhibits profound drought tolerance, rendering it an excellent candidate for the increasingly arid, low-precipitation summer conditions observed globally¹. Furthermore, cultivating cardoon offers significant secondary economic benefits; beyond cheese coagulation, the seeds yield up to 32.47% oil (rich in linoleic and oleic acids) suitable for biodiesel production, while the stalks provide valuable lignocellulosic biomass⁶.

4. Structural Botany and Phenological Harvesting Protocols

The efficacy of a botanical coagulant is inextricably linked to the physiological maturity and structural botany of the source plant at the precise moment of harvest. The concentration and functional ratio of active proteases fluctuate significantly across the plant's life cycle, dictating that harvesting protocols must be governed by strict phenological markers.

4.1 The Asteraceae Matrix: *Cynara cardunculus*

Cynara cardunculus can reach a height of 3 meters and produce up to 15 inflorescences per plant⁶. The critical aspartic proteases responsible for milk coagulation—designated as cardosins and cyprosins—are not distributed uniformly throughout the plant tissue. They are highly localized, accumulating predominantly in the protein storage vacuoles of the stigmatic papillae and the extracellular matrix of the stylar transmitting tissue located exclusively in the violet pistils of the flower⁶. Consequently, harvesting must focus entirely on the upper violet fraction (styles and stigmas) of the capitulum⁶.

The optimal harvest window is governed by the BBCH-scale for phenological growth. Extraction potential peaks during the full flowering stage (BBCH stage 625 to 629), when the secondary inflorescences have at least 50% of their flower petals visible, are fully open, and express a vivid blue-purple pigmentation⁶. Harvesting premature flowers results in an unacceptably low concentration of active aspartic proteinases, while harvesting post-senescence flowers (BBCH stage 800 and beyond, when the capitulum turns brown and pappus becomes visible) yields heavily degraded enzymes with poor milk-clotting capacity⁶.

Furthermore, the genetic ecotype of the *C. cardunculus* plant fundamentally alters the biochemical profile of the resulting extract. Extensive chromatographic profiling of different accessions reveals distinct biochemical phenotypes that directly predict the final texture of the cheese. For instance, the "6M" ecotype exhibits a high total concentration of proteases with a

clear prevalence of Cardosin A (a highly specific, chymosin-like enzyme) and vigorous ^K

-casein action, yielding cheeses with a firmer paste²⁰. Conversely, the "3M" ecotype demonstrates an absolute absence of Cardosin A, relying entirely on alternative proteases (like Cardosin B) for coagulation. Extracts from the 3M ecotype display a slow micellar aggregation rate and aggressive β -casein degradation, which results in remarkably soft, nearly liquid cheese textures²⁰. The physical harvest is performed manually using sterilized, long-bladed scissors to excise the entire inflorescence, after which the violet pistils are meticulously separated by hand from the receptacle and non-active straw to prevent organic contamination⁶.

4.2 The Urticaceae Matrix: *Urtica dioica*

Unlike the highly localized floral proteases of the cardoon, the milk-clotting enzymes of the stinging nettle (*Urtica dioica*) are distributed throughout the foliar tissue (leaves) of the plant¹. Nettle leaves possess a complex repertoire of proteases alongside robust defense proteins, such as *Urtica dioica* agglutinin (UDA), a heat- and acid-resistant lectin¹.

Harvesting *U. dioica* presents unique logistical challenges due to the plant's evolutionary defense mechanisms. The foliar surfaces are densely populated with trichomes (stinging hairs) that act as hypodermic needles, injecting a potent biochemical cocktail of histamine, acetylcholine, and formic acid upon contact¹. Harvesting requires advanced mechanical protection to prevent severe contact dermatitis. The aerial leaves should be harvested in the late spring to early summer, prior to the plant transitioning into its flowering phase, ensuring maximum protein retention and protease activity within the leaf structures.

5. Thermodynamics of Dehydration and Cellular Disruption

Following the harvest, the raw botanical material must be immediately stabilized. The primary objectives are to halt endogenous enzymatic auto-degradation, prevent fungal proliferation, and standardize the moisture content for accurate dosing during the subsequent aqueous extraction. The thermodynamic parameters of this dehydration phase exert a profound influence on the ultimate enzymatic titer of the biomaterial.

5.1 Air Dehydration vs. Forced-Convection in Cardoon Pistils

The traditional preservation methodology for *C. cardunculus* across the Iberian Peninsula relies on passive air dehydration. Freshly separated purple styles and stigmas are spread in thin layers in shaded, well-ventilated environments at ambient room temperature (approximately 20°C to 30°C) for an extended duration of 30 to 60 days⁶. The biomaterial is subjected to regular physical turnings to facilitate uniform moisture evaporation and prevent localized anaerobic zones that promote mold⁶.

While this traditional, slow-drying protocol succeeds in preserving the flowers for year-round cheesemaking, it is highly deleterious to the enzymatic payload. Prolonged exposure to oxygen, ambient light, and fluctuating humidity during the 30- to 60-day drying period results in severe auto-proteolysis and oxidative degradation of the cardosin structures⁶. Empirical analyses demonstrate that traditional slow drying destroys up to 75% of the potential enzymatic coagulant available in the fresh flower (expressed per unit of dry matter), significantly depressing the

specific Milk-Clotting Activity (MCA)⁶.

To circumvent these catastrophic enzyme losses, modern dairy biotechnologists have engineered controlled, fast-drying protocols. Utilizing forced-convection drying chambers equipped with active dehumidification and ultraviolet (UV) sanitization, the drying time for cardoon pistils can be aggressively reduced from several weeks down to merely 12 hours²⁶. Operating these chambers at a highly controlled, moderate temperature of 35°C accelerates the

reduction of water activity (a_w) to below 0.500, rapidly crossing the threshold where metabolic and microbial degradation ceases²⁶.

Remarkably, a controlled elevation of the drying temperature does not inherently denature the cardosins if the moisture is removed rapidly. Research indicates that subjecting the pistils to 50°C to 55°C for 5 days yields an MCA that is approximately 17% higher (on a dry matter basis) than the traditional 30-day room-temperature method⁶. Conversely, extreme thermal interventions, such as rapid dehydration at 100°C for 5 hours, induce structural unfolding of the proteinases, resulting in a net loss of activity⁶.

For absolute preservation, lyophilization (freeze-drying) represents the apex of dehydration technology. Plunging the fresh pistils into liquid nitrogen and subsequently sublimating the ice under a deep vacuum (-55°C at 0.09 mPa for 7 hours) perfectly preserves the tertiary structure of the aspartic proteases, yielding a highly potent, stable enzyme powder²⁸. While technologically superior, the intensive energy costs of lyophilization generally relegate its use to laboratory settings and the production of standardized commercial plant rennet powders.

5.2 Dehydration and High-Pressure Disruption of *Urtica dioica*

The processing of *Urtica dioica* leaves follows a similar imperative for rapid moisture reduction. Fresh nettle leaves possess an exceedingly high water content (65% to 90%) and must be dried quickly at low temperatures to prevent the loss of active proteinaceous compounds¹⁴.

Once dried, the brittle nettle leaves present an extraction barrier. Because the active proteases in *U. dioica* are bound within robust cellulosic plant cell walls and intracellular compartments, simple steeping is often insufficient for maximal enzyme recovery. Advanced preparation protocols employ High-Pressure Homogenization (HPH) or Pulsed Electric Fields (PEF) to induce severe mechanical and electrical cellular disruption prior to extraction²³. HPH involves forcing a hydrated dispersion of nettle powder (e.g., a 5% w/w solution) through a two-stage homogenizer at extreme pressures (300 to 600 bars)²³. This process effectively shatters the cell

membranes, pushing the particle size distribution down to a D_{90} of less than 150 μm while ensuring the temperature does not exceed 40°C²³. This pre-extraction mechanical processing increases total extractable protein yields to 11.60%, vastly outperforming passive diffusion methods²³.

Dehydration & Processing Parameter	Applied Species	Thermodynamic Protocol	Impact on Enzyme Kinetics & Yield
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Traditional Air Drying	<i>Cynara cardunculus</i>	20-30°C for 30-60 days in ambient shade ⁶ .	High rate of enzyme loss (up to 75% MCA reduction) due to prolonged auto-proteolysis ⁶ .
Forced Convection (Fast Drying)	<i>Cynara cardunculus</i>	35°C for 12 hours (pistils) with active dehumidification ²⁶ .	Rapid drop in water activity ($a_w < \quad$) highly preserves active catalytic sites and maximizes yield ²⁶ .
Lyophilization (Freeze-Drying)	Both species	Freezing in liquid nitrogen; sublimation at -55°C and 0.09 mPa ²⁹ .	Near-perfect preservation of molecular structure; zero thermal denaturation, maximizing shelf-life ²⁸ .
High-Pressure Homogenization (HPH)	<i>Urtica dioica</i>	Mechanical shearing at 300-600 bars, maintaining temp < 40°C ²³ .	Shatters cellulosic cell walls, increasing total protein extraction yield up to 11.60% ²³ .

6. Aqueous Extraction: The Biochemical "Brewing" of Botanical Coagulants

The transition from a stabilized, dried botanical matrix to an active milk-coagulant liquid requires an aqueous extraction phase—colloquially referred to as "brewing the tea." This is arguably the most biochemically sensitive stage of the preparation process. The solubility, shelf-stability, and specific catalytic activity of the resulting enzymes are strictly governed by the physical maceration, pH, ionic strength, temperature, and duration of the steep.

6.1 Maceration Kinetics and "Tea" Recipes for Cardoon Pistils

The traditional artisanal extraction of *C. cardunculus* is a cold-water infusion. The paramount rule of botanical extraction is to avoid heat; utilizing hot water to "brew" the coagulant, as one might for a beverage tea, will instantly denature the cardosins and completely destroy the coagulant¹.

The standard application ratio for cardoon extraction is highly concentrated. To coagulate a standard vat, cheesemakers employ a ratio equating to 1 gram of dried floral pistils to coagulate 1 liter of milk³⁰. To prepare the extract, the dried pistils are first subjected to mechanical maceration. Typically, the dried stamens are ground to a coarse powder utilizing a mortar and pestle or a mechanical blender²⁵.

The Standard Extraction Recipe: The ground pistils are suspended in distilled or tap water at a ratio of 1 gram of pistils to 10 mL of water (e.g., 35 grams of ground pistils soaked in 500 mL of water)³⁰. The suspension is allowed to steep in the dark at room temperature (approximately 15°C to 25°C) for a duration ranging from 30 minutes for rapid extractions up to 24 hours for maximum protein yield³⁰. After the maceration period, the dark, highly aromatic liquid is filtered through a standard paper filter or fine cheesecloth to remove the physical plant matter, yielding the crude enzymatic extract²⁶. The filtered extract is then added directly to the ripened cheese milk (typically at 30°C) at a ratio of 100 mL of extract solution per 10 liters of milk³⁰.

From a strict biochemical perspective, pure aqueous extraction is sub-optimal for industrial standardization. The solubility of the heavy and light chains of Cardosin A and Cardosin B is significantly enhanced by utilizing a highly controlled buffer system rather than plain water. Suspending the ground pistils in a 0.1 M phosphate-citrate or sodium acetate buffer adjusted to a slightly acidic pH of 5.0 to 5.5 profoundly stabilizes the enzymes²⁵. Furthermore, the addition of complexing agents, such as 5 mM EDTA, during extraction actively chelates divalent cations, preventing the precipitation of the target proteins and deactivating endogenous metalloproteases that might otherwise degrade the cardosins before they reach the cheese vat²⁹.

6.2 pH-Dependent Catalysis and Colloidal Stability in *Urtica dioica*

Extracting the active proteases from *Urtica dioica* reveals a complex thermodynamic conflict between catalytic optimization and colloidal stability. For fresh cheese production, historical artisanal recipes suggest adding approximately 20 grams of fresh nettle leaves directly to 2 liters of whole cow's milk³⁵. However, modern extraction aims to isolate the enzymes into a concentrated liquid.

When preparing an aqueous nettle extract, the pH of the extraction buffer dictates the success of the process. Analytical studies demonstrate that nettle proteases exhibit their absolute maximum specific milk-clotting activity (0.021 U/mL) and highest general proteolytic capability when extracted in a highly acidic environment, specifically at pH 4.0¹.

However, exploiting this highly acidic extraction parameter introduces severe physical instability to the extract itself. Non-destructive optical monitoring utilizing Turbiscan stability indexing demonstrates that nettle extracts prepared in the pH 3.0 to 4.0 range exhibit massive colloidal instability¹. At these low pH levels, the Turbiscan Stability Index (TSI) approaches a value of 20, indicating rapid particle aggregation, massive sedimentation, and phase separation of the chlorophyll residues and extracted proteins¹. If this highly acidic extract is not utilized immediately, the coagulating enzymes precipitate out of solution, rendering the extract functionally useless for commercial distribution.

Conversely, extracting *U. dioica* at a nearly neutral pH (5.0 to 6.5) reverses this dynamic. The

resulting fluid is highly stable, maintaining a homogenous suspension with a very low TSI of approximately 1, allowing for prolonged storage¹. Yet, this physical stability comes at the severe cost of depressed catalytic efficiency¹. The dairy technologist must therefore calculate a precise compromise: either extract at pH 4.0 and deploy the coagulant immediately inline into the cheese milk, or extract at pH 5.5 to allow for a commercially viable shelf-life of the liquid extract, accepting that a higher volumetric dosage will be required in the cheese vat.

7. Structural Biology and Catalytic Kinetics of Plant Proteases

Once the botanical extract is introduced to the cheese milk, the resulting biochemical cascade is fundamentally defined by the specific structural biology and kinetic parameters of the constituent plant proteases.

7.1 The Aspartic Proteases of *Cynara cardunculus*

The milk-clotting efficacy of *C. cardunculus* is driven by its high concentration of aspartic proteinases (APs), specifically Cardosin A and Cardosin B¹. These enzymes belong to the pepsin family (clan AA, family A1) and are synthesized in the plant as inactive zymogens (preproenzymes)¹. They contain a signal peptide for endoplasmic reticulum translocation, a prosegment for correct folding, and a unique 104-amino acid Plant-Specific Insert (PSI)¹. During maturation and trafficking to the vacuole, the PSI and prosegment are excised¹. The mature enzymes are heterodimeric glycoproteins composed of a heavy chain (approximately 31 to 34 kDa) and a light chain (14 to 16 kDa) linked by hydrophobic interactions and disulfide bonds¹. The catalytic mechanism relies on two critical aspartate residues—Asp80 and Asp267—located at the base of a deep active site cleft, which activate a water molecule to perform a nucleophilic attack on the target peptide bond¹.

Cardosin A represents the apex of botanical specificity. Kinetically, it acts as a nearly perfect ortholog to calf chymosin, demonstrating a massive catalytic preference for the critical

$Phe_{105} - Met_{106}$ bond of κ -casein¹. This highly specific cleavage rapidly releases the CMP, destabilizing the micelle and inducing aggregation with minimal damage to the structural core of the micelle¹.

Conversely, Cardosin B functions more akin to mammalian pepsin¹. While it successfully

cleaves κ -casein (and can also cleave it at $Ser_{104} - Phe_{105}$), it possesses a significantly

broader sequence selectivity⁴⁰. Cardosin B aggressively attacks the internal α_{s1} - and β

-casein fractions, cleaving bonds such as $Leu_{192} - Tyr_{193}$ in β -casein¹. Kinetic modeling

of the specificity ratio (k_{cat}/K_m) indicates that the dynamic ratio of Cardosin A to Cardosin B within the crude extract fundamentally dictates the rheological behavior and final texture of the resulting cheese²⁰.

7.2 The Cysteine Proteases of *Urtica dioica*

The coagulating mechanisms of *Urtica dioica* are primarily driven by potent cysteine proteases. Detailed purification and characterization of nettle extracts have isolated specific enzymes, such as a 33 kDa cysteine protease (KgCP) that exhibits remarkable catalytic performance²². This

specific nettle protease operates with a V_{max} of 103.7 U/min and a K_m of 0.025 μmol , functioning optimally at 55°C and pH 5.5²².

Unlike the highly specific aspartic proteases of the cardoon, nettle proteases induce a rapid, non-specific hydrolysis of the κ -casein barrier alongside broader protein degradation²².

Because the proteolysis is less targeted, nettle extracts are optimally suited for the production of fresh, rapid-consumption cheeses where long-term structural integrity during ripening is not required⁴⁵. Fascinatingly, nettle extracts have shown remarkably high specific clotting yields when applied to camel milk, vastly outperforming traditional bovine chymosin, presenting a unique technological niche for dromedary dairy processing³⁶.

8. Coagulation Dynamics, Rheology, and Cheese Yield

The physical consequences of a coagulant's enzymatic specificity are objectively quantified through dynamic rheological monitoring and final cheese yield calculations. The paramount metric for evaluating a botanical coagulant is its Milk-Clotting Activity (MCA) relative to its general Proteolytic Activity (PA), expressed as the MCA/PA ratio¹.

8.1 Rheological Firmness (G')

The formation of the cheese curd is dynamically monitored by measuring the storage modulus (G'), which represents the elastic strength and firmness of the gel network, and the loss

modulus (G''), representing the viscous properties¹.

Because plant proteases—especially Cardosin B and the cysteine proteases of nettle—engage in higher rates of non-specific background proteolysis, they simultaneously build and degrade

the casein matrix. Consequently, the G' for gels induced by botanical extracts is frequently lower than those induced by pure animal chymosin, resulting in softer, more fragile curds with

lower apparent yield stress¹. The aggressive cleavage of the α_{s1} - and β -casein fractions physically disrupts the three-dimensional gel network before it can fully stabilize¹.

8.2 Dry Matter Yield and Milk Sourcing

The unspecific proteolysis inherent to botanical coagulants has direct economic implications for dry matter yield. The broad cleavage patterns break the structural caseins into smaller, soluble peptides that fail to integrate into the curd matrix¹. These peptides, along with non-protein nitrogen (in urea form), are expelled into the whey stream during syneresis, representing a direct loss of total protein retention in the cheese¹.

To offset this yield loss, the selection of the milk source is critical. Ovine (sheep) milk is the traditional substrate for cardoon coagulants, and for precise biochemical reasons. Ovine milk is remarkably rich in total solids, possessing approximately 7.5% fat and 4.6% caseins, compared

to bovine milk which averages 3.9% fat and 2.6% caseins⁵⁰. Consequently, ovine milk produces a vastly superior cheese yield: it requires only about 5.5 liters of ewe's milk to produce 1 kg of cheese, whereas it requires approximately 11 liters of cow's milk to yield the same mass⁵⁰. The dense protein matrix of ovine milk provides a higher substrate concentration, buffering the aggressive proteolytic action of the cardosins and ensuring a structurally viable curd despite the elevated PA⁵⁰. Furthermore, the specific cleavage of ovine α_{s1} -casein at the *Leu*₉₉ – *Arg*₁₀₀ bond by cardosins yields unique texture profiles not reproducible in bovine matrices⁵⁰.

9. Downstream Matrix Modulation and the Mitigation of Bitterness

The most profound technological hurdle associated with botanical coagulants is the development of aggressive bitterness during the cheese ripening phase¹. The broad-spectrum cleavage of α_{s1} - and β -casein by enzymes like Cardosin B generates an accumulation of low-molecular-weight, highly hydrophobic, proline-rich oligopeptides¹. These peptides persist in the cheese matrix and strongly bind to human bitter taste receptors, yielding a highly astringent and commercially unacceptable flavor profile in aged cheeses¹. To integrate botanical coagulants into commercial production, the dairy technologist must deploy specific downstream interventions.

9.1 Physical and Chemical Interventions

One highly effective physical strategy involves the pre-treatment of the cheese milk via Ultrafiltration (UF)¹. By utilizing UF membranes to concentrate the total solids, fat, and native caseins of the milk (at a low concentration factor, e.g., 1.2x) prior to renneting, the technologist fundamentally alters the enzyme-to-substrate ratio¹. The UF-concentrated matrix exhibits a significantly higher chemical buffering capacity, which physically impedes the excessive secondary proteolysis of the cardosins, effectively reducing Residual Chymosin Activity (RCA) per gram of protein and yielding firmer curds with substantially mitigated bitterness¹.

Modifying the brining parameters offers direct chemical control. Plant proteases generally exhibit lower sensitivity to saline environments than chymosin; however, strategically elevating the NaCl concentration or extending the salting time directly limits ongoing microbial and enzymatic proteolysis¹. For example, NaCl concentrations above 5% significantly decrease casein

proteolysis, and at 20% NaCl, the proteolysis of β -casein by cardosins is almost completely inhibited¹⁹. This targeted deceleration during prolonged aging prevents the threshold accumulation of bitter peptides¹.

9.2 Microbiome Engineering via *Lactobacillus helveticus*

The most elegant solution to botanical bitterness is biological intervention via microbiome engineering. While botanical proteases cleave caseins into long, hydrophobic, bitter oligopeptides, these structures are highly resistant to standard proteolytic degradation⁵². The

release of the amino acid proline from these peptides requires highly specific proline-targeted peptidases⁵².

To achieve this, technologists introduce highly specialized strains of *Lactobacillus helveticus* (such as strains LH-B02 or CNRZ 32) into the cheese milk as a Non-Starter Lactic Acid Bacterium (NSLAB) adjunct culture⁵². *L. helveticus* is largely deficient in endogenous amino acid biosynthesis and must scavenge them from its environment⁵². To survive the hostile cheese matrix, it expresses a robust arsenal of proline-specific peptidases (including PepI, PepP, PepQ, and PepX)⁵².

As the cardosins generate the bitter hydrophobic peptides, the *L. helveticus* peptidases rapidly hydrolyze them, shearing the proline bonds and reducing the bitter compounds into flavor-neutral, free amino acids⁵². This symbiotic interplay perfectly demonstrates the nuanced complexity of artisanal cheesemaking: a plant-derived enzyme is leveraged for initial coagulation, while a highly selected bacterial adjunct is deployed to biochemically "clean up" the resultant proteolytic excess, yielding a perfectly balanced, non-bitter, entirely vegetarian cheese. Additionally, this intense secondary proteolysis frequently releases bioactive peptides—such as isracidin—which possess potent antimicrobial, antihypertensive, and antioxidant properties, elevating the nutritional profile of the cheese⁵⁵.

10. Whey Valorization and By-Product Economics

The high proteolytic activity of plant coagulants deeply impacts the composition of the resulting whey, presenting unique opportunities for by-product valorization¹. Whey represents 85% to 95% of the total milk volume utilized in the vat and retains approximately 55% of the milk's original nutrients, including 20% of the proteins¹.

When utilizing *C. cardunculus* or *U. dioica*, the resulting liquid is classified as "Sweet Whey," produced at a pH of approximately 6.0 to 6.7¹. Because botanical proteases cleave higher amounts of non-protein nitrogen and specific caseinomacropptides into the whey compared to chymosin, this sweet whey is incredibly rich in soluble proteins and lactose (46–52 g/L)¹. High lactose levels contribute to extreme Biological Oxygen Demand (BOD), classifying untreated whey effluent as a severe environmental pollutant¹.

To valorize this waste stream, the cardoon-coagulated sweet whey is traditionally heated to near-boiling temperatures to thermally denature and precipitate the remaining whey proteins

(primarily β -lactoglobulin and α -lactalbumin), producing high-value secondary whey cheeses such as Portuguese Requeijão or Italian Ricotta¹. The residual liquid remaining after Requeijão production, known as Second Cheese Whey (SCW) or *Sorelho*, still contains massive amounts of lactose. This SCW can be processed via industrial nanofiltration for carbohydrate recovery or dried into Whey Protein Isolate (WPI) powders for sports nutrition, effectively closing the economic loop of the cheesemaking process¹.

11. Precision Fermentation and Recombinant Expression

To satisfy the demands of the modern dairy industry for absolute standardization without

sacrificing the "vegetarian" label or the unique cleavage profiles of plant enzymes, biotechnology has increasingly looked to precision fermentation to express botanical coagulants, bypassing the physical plant entirely¹.

Just as the gene for calf chymosin was cloned into microorganisms in the 1990s to create Fermentation-Produced Chymosin (FPC), researchers have successfully mapped and expressed the genes for Cardosin A, Cardosin B, and Progaline B in eukaryotic microbial hosts such as *Kluyveromyces lactis* and *Pichia pastoris*¹.

Expressing Cardosin B in yeast involves complex genetic engineering. The native gene must be modified to remove the Plant-Specific Insert (PSI)—a 104-amino acid domain that normally directs the enzyme to the plant vacuole—because it severely hampers cellular secretion in yeast expression systems¹. In recombinant models, the PSI is replaced with a small synthetic linker sequence, such as a simple three-glycine (GGG) chain connecting the heavy and light chains¹.

Fascinatingly, this synthetic recombinant Cardosin B exhibits a more stringent sequence selectivity and a lower general catalytic efficiency compared to the wild-type plant enzyme¹. From an industrial perspective, this is highly advantageous: it effectively eliminates the excessive background proteolysis associated with crude thistle extracts¹. This allows for the reliable, continuous production of firm, non-bitter cheeses while retaining the functional properties and consumer-friendly "plant-derived" narrative of the enzyme¹. However, because European artisanal markets exhibit high resistance to Genetically Modified Organisms (GMOs), producers seeking to market authentic, hyper-regional, terroir-driven PDO cheeses will continue to rely on the agricultural cultivation and precise aqueous extraction of the physical flowers¹.

12. Strategic Conclusions

The deployment of *Cynara cardunculus* and *Urtica dioica* as botanical milk coagulants transcends simple ingredient substitution. It represents a multidisciplinary endeavor requiring mastery of protein biochemistry, adaptation to evolving climatic zones, and rigorous adherence to food safety protocols.

1. **Phenological Precision:** The harvest of *Cynara cardunculus* must be tightly synchronized with the BBCH growth scale to capture peak Cardosin A/B ratios within the stigmatic papillae. Harvesting is not a generic agricultural task, but the first step in defining the eventual rheological firmness (G') of the cheese matrix.
2. **Thermodynamic Dehydration:** Traditional 30-day air drying is biochemically destructive, inducing massive auto-proteolytic losses (up to 75%). The adoption of 35°C forced-convection drying or lyophilization drastically preserves the structural integrity and catalytic potential of the enzymes, maximizing processing efficiency.
3. **Electrochemical Extraction ("Brewing"):** The aqueous maceration of the dried botanicals must be carefully buffered and precisely timed. Extracting *U. dioica* at pH 4.0 maximizes absolute proteolytic yield but induces severe colloidal instability (TSI ~20), demanding immediate utilization or rigorous pH balancing to ensure extract viability. The traditional cardoon extraction of 1 gram of pistils per 10 mL of water remains standard but benefits vastly from buffer stabilization.

4. **Zoonotic Exclusion via Microfiltration:** The severe threat of *Echinococcus multilocularis* contamination absolutely precludes the use of raw, unfiltered wild extracts. Because the thermal lability of the cardosins and nettle proteases prevents standard heat pasteurization without destroying the coagulant, cold processing through a 20 μm or 0.22 μm PES membrane is a non-negotiable processing requirement to guarantee biological safety.
5. **Matrix Modulation for Bitterness:** The inherently broad proteolytic cleavage specificities of Cardosin B and nettle extracts invariably lead to the generation of bitter, proline-rich hydrophobic peptides from α_{s1} - and β -caseins. The strategic integration of *Lactobacillus helveticus* as an NSLAB adjunct culture perfectly counters this defect, utilizing targeted peptidolytic pathways to degrade the bitter compounds into neutral amino acids, ensuring the commercial viability of aged botanical cheeses.

Through the rigorous application of these agronomic, thermodynamic, filtration, and microbiological controls, dairy technologists can successfully harness the profound catalytic power of the thistle and the nettle, producing artisanal, clean-label dairy products of the highest organoleptic quality.

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