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Chapter 5

Cynara cardunculus: Use in Cheesemaking and Pharmaceutical Applications

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Abstract

Cynara cardunculus L. is the most widespread species of *Cynara* genus (f. *Asteraceae*). This herbaceous perennial plant is native to the Mediterranean region and invasive in other parts of the world, growing naturally in harsh habitat conditions. There are three subspecies: globe artichoke; cultivated cardoon and the progenitor of the two, the wild cardoon. The culture of *Cynara cardunculus* L. follows an annual growth cycle, emerging in autumn and harvesting in summer. *Cynara cardunculus* has been considered as a multi-purpose crop due to its relevant biochemical profiles. Inflorescences have been used as food, whereas leaves are a rich source of bioactive compounds. Consequently, larger plants without spines have been selected for technological purposes. Due to its high cellulose and hemicellulose content, the lignocellulosic fraction has been used as solid biofuel, biogas and bioethanol. Both pulp fibers production and seeds oil are suitable for biodiesel production. Over the centuries, the inflorescence pistils of *Cynara cardunculus* L. have been widely used for cheesemaking. The present chapter gives an overview of the *Cynara cardunculus* L. emphasizing recent knowledge regarding the use, conservation, preparation and application of *Cynara cardunculus* in ovine milk cheesemaking, as well as other biotechnological applications.

Keywords: cardoon, *Cynara cardunculus* L., pistilis, vegetable coagulant, cardosins, bioactive compounds, ovine milk cheese

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1. Introduction

Cynara cardunculus L. is a herbaceous perennial diploid plant. It is the most widespread species of the genus *Cynara* and belongs to the Asteraceae family. Recent revisions about *Cynara* genus classification have sparked discussion over whether wild cardoon (*C. cardunculus* var. *sylvestris*), cultivated cardoon (*C. cardunculus* var. *altilis*) and globe artichoke (*C. cardunculus* var. *scolymus* L.) should be classified as different species, or as subspecies [1]. Studies about morphology and phytogeography of the *Cynara* genus supports that the referred plants belongs to a single species and should consequently be classified as subspecies [2–8]. Regardless the subspecies, cardoon is part of Mediterranean flora, distributed throughout the Mediterranean basin, Macaronesia (Madeira and Canary Islands), North Africa, Cyprus and Turkey [2, 9–11], is also a colonizer in Mexico, California, Argentina, Chile, Peru, Australia, China and West Africa [11–13]. Regarding artichoke, its production is worldwide disseminated, with great economic impact, especially in Italy, Spain, France and Turkey [12]. The production of cultivated cardoon seems to be more restricted to South Europe, namely in Spain, Italy, France, Greece and Portugal [13–17].

Wild cardoon grows spontaneously in marginal areas of field crops, pastures and along paths in dry areas and in soils of various characteristics. The plant, either cultivated or wild, can persist for a number of years, over 10 years, re-sprouting annually from its large perennial taproot [18, 19]. New seedlings usually germinate after the autumn rains, then first cotyledons emerge, growing slowly through in a rosette arrangement. Cardoon plants hold in the rosette stage during winter and early spring, when stalks start to elongate. As the flower stems develop, the lower rosette leaves begin to die off. Plants usually flower in the early summer, followed by the dieback of their aerial growth. Seedlings do not generally flower in their first year, as their energy is absorbed on the development of its deep taproot. New growth occurs with the autumn rains, and the cycle starts over [16].

Both plant agronomic characteristics and human selection of certain phenotypes, over the years, can possibly explain the use specificity, of each subspecies, for different purposes. *C. cardunculus* L. species lignocellulosic fraction shows great potential as solid biofuel [14], as well as for biogas production [20, 21] and bioethanol [22, 23]. *C. cardunculus* L. biomass productions can reach 14–20 t DW/ha per year, while according the studies of Pesce et al. [24], the wild cardoon accession was less productive in terms of biomass accumulation (11.8 t DW/ha per year) compared to cultivated forms (*Altilis* 41′ and 'Bianco avorio') producing, respectively, 19.1 t and 16.8 t DW/ha per year. Stalks and capitula of cultivated cardoon may also be used to produce pulp fibers [14, 25, 26]. In Southern Portugal, a region characterized by very hot and dry summers, a large scale cultivation of the cardoon *C. cardunculus* L. for biomass production was installed in a total of 77.4 ha. The field biomass yield was estimated at 7.5 t/ha, and the seed yield was 603 kg/ha after the second growing season [16].

C. cardunculus seed's oil fatty acid composition has also revealed a great potential for biodiesel production [14, 27]. *C. cardunculus* seed yield, and see oil composition is quite similar to sunflower oil. *Cynara* crop seed yield has been estimated at 1.36 t/ha per year [28], and a maximum seed oil content of 32.47% has been described by Curt et al. [29], while oil composition is on average 11% palmitic, 4% stearic, 25% oleic and 60% linoleic fatty acids.

Artichoke extracts make part of commercial dietary juices and capsules for digestion dysfunction treatment, being related with the bioactive extractives composition [30]. Nevertheless, cultivated cardoon leaves can be a good substitute for green forage during wintertime [14], and a great biomass source of the sesquiterpenic lactone cynaropicrin [31]. Moreover *C. cardunculus* L. presents diverse nutraceutical, pharmacologic and/or therapeutic properties [32–38].

In Portugal, *C. cardunculus* is known as "cardo de coalho" or "cardo hortense" [39, 40]. The richness of inflorescence pistils in aspartic proteases, named cardosins, has potentiated its wide use for cheesemaking for centuries [41]. Aqueous extracts of *C. cardunculus* pistils have been successfully employed for centuries in the manufacture of French, Italian, Spanish and Portuguese cheeses, and legally required to manufacture a number of protected designation of origin (PDO) cheeses in Portugal and Spain. The coagulant enzymes extracted from *C. cardunculus L.* flowers, cardosins, are aspartic proteinases, which have been assigned specific and technologic consequences, namely in sheep cheeses [42]. In cheesemaking, these enzymes show a similar action to other aspartic proteinases used in cheesemaking, such as chymosin. Cleaving the Phe105-Met106 binding of ovine and bovine κ-caseins [43–45] allowing the casein micelles aggregation by the milk ionic calcium. Cardosins reveal a more intense secondary proteolytic action on cheese α_s- and β-casein than other coagulants, with impact on the cheeses biochemical and sensory properties.

The most recent knowledge regarding the use, conservation, preparation and application of *Cynara cardunculus* plants in cheesemaking and others biotechnological applications are reviewed in the present chapter.

2. The species *Cynara cardunculus* L.

2.1. Origin and population structure

The taxonomy on the gender *Cynara* has evolved over time, and it seems to exist some confusion about the type of plants that fall under the designation of *C. cardunculus* L. species; even for the use in cheese manufacture, the descriptions of plants have been a little variable. In fact, the definition of the species that comprises the genus *Cynara* is somewhat complex, because some of them take different names, appearing eventually placed in other genera, with which there are affinities. For example, the number of species considered in the genus review of Wiklund [2] is eight, whereas the previous treatment of the same genus, in 1838, considered only three.

Illustrating the designations complexity or classification, Bailey and Bailey [46] refer to 10 Mediterranean species, 7 of which are referred to by Tutin et al. [47] as European: *C. scolymus*, *C. tournefortii*, *C. humilis*, *C. cardunculus*, *C. cornigera*, *C. alba* and *C. algarbiensis*. However, the species *C. humilis* is often identified as belonging to the genus *Bourgea* (*Bourgea humilis* L.) [2, 48] and *C. alba* corresponds, according to Valdes et al. [48], to the species *C. baetica*, also referred as belonging to the genus *Cirsium*. From this, one species, *Cirsium vulgare*, was used by Wiklund [2] as a test for its study of the genus *Cynara*. *C. scolymus* is referred to as spontaneously unknown by Tutin et al. [47] and Franco [49]. Moreover, Foury [3] distinguish only three species: (i) *C. cardunculus*, whose distribution coincides with that found by Wiklund [2]

for *C. cardunculus* spp. *flavescens* (the NW Mediterranean region, whereas *C. cardunculus* spp. *Cardunculus* generally occurs in regions with coastal influence, Macaronesia, Portugal, Central and NE Mediterranean distribution); (ii) *C. sibthorpiana* (Greece, Cyprus and Crete), designated by Tutin et al. [47] by *C. cornigera* and (iii) *C. syriaca*, in Palestine. Valdés et al. [48] distinguish *C. cardunculus* L., *C. humilis* L., *C. algarbiensis* and *C. baetica* in Andaluzia. Franco [49], in Portugal flora, refers to *C. scolymus*, *C. cardunculus*, *C. humilis*, *C. algarbiensis* and *C. tournefortii*, the last one being excluded of *Cynara* genus by Wiklund [2].

The recent revisions on *Cynara* genus classification have sparked discussion over whether the wild cardoon (*C. cardunculus* var. *sylvestris*), cultivated cardoon (*C. cardunculus* var. *altilis*) and globe artichoke (*C. cardunculus* var. *scolymus* L.) should be classified as different species, or as subspecies [50]. According to the results of Gatto et al. [12], the origin of these forms may have new scenarios: (i) the globe artichoke was domesticated a long time ago from wild material in Sicily/northern Africa; (ii) the leafy cardoon was derived from western Mediterranean (Portugal, Spain) wild cardoon and (iii) the eastern wild cardoon from Italy, Greece, Tunisia and Malta represents the only original wild form, that gave origin to both globe artichoke and cultivated cardoon. Furthermore, the leafy cardoon might have returned to wild forms, giving rise to the so-called western wild cardoon (Spain, Portugal). So, plant types considered before as different species or varieties, as *C. scolymus* L., cultivated for artichoke, and *C. cardunculus* L. var. *altilis* DC, known for its juicy young leaves [4], or *C. cardunculus* L. subsp. *cardunculus* and *C. cardunculus* L. subsp. *flavescens* [2], with differences on size, leaves and flowers of plants, and on spinyness of bracts, are considered now as coming from the perennial wild, *C. cardunculus* L. var. *sylvestris* (Lam.).

All these different forms of plants belong actually to a single species, *C. cardunculus* L. [4]. Studies about morphology and phytogeography of the *Cynara* genus support that the referred plants belong to a single species and should consequently be classified as subspecies [2–8]. The variability of characteristics within the species is a conclusion drawn by different authors [2, 3] and could be observed before in Portugal by Morbey [51], who collected plants of *C. cardunculus* prior to settle of experimental fields. However, despite the richness of wild cardoon germplasm, the identification and characterization of its genetic resources are scarcely investigated. In Portugal, *C. cardunculus* L. has an increasingly limited distribution, becoming restricted to certain areas where it has benefited of some care, even being sometimes cultivated [52].

Molecular data provided evidence that the western wild cardoon, the *C. cardunculus* L. subsp. *flavescens*, distributed in Spain and Portugal and characterized by more robust plants, is genetically closer related to cultivated cardoon, while the eastern wild cardoon, the *C. cardunculus L.* subsp. *cardunculus*, might be the progenitor of the globe artichoke [1, 7, 50], confirming the conclusions of recent revisions of *Cynara* genus. The two crops have possibly been derived from human pressure selection for either large non-spiny heads on one side, or non-spiny large stalked tender leaves on the other side [1, 5]. In the last decades, several molecular markers (RAPD, AFLP, SSR and ISSR) have been used for *C. cardunculus* population characterization. Among genetic markers available, the simple sequence repeats (SSR) are highly informative since they are codominant and generally highly polymorphic [53]. Recent studies have been conducted in Tunisia by Ben Ammar et al. [54] and Khaldi et al. [55], which revealed a large variability among wild cardoon populations.

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The agronomic plant characteristics, combined with human selection over the years, possibly explain the specificity of the use of each subspecies for each different purpose. Different parts of the plant, such as leaves and inflorescences, with high relevant biochemical profiles, are used as food providing the selection for larger, tender and non-spiny plants [12, 56].

2.2. Historical and etymological archives

Throughout history, cardoon use had curious applications including torment weapon, confectionery, medicine, besides its role as a coagulant in cheesemaking. According to Barreira [57], the reference of cardoon in the Bible is associated with "torment" or "suffering", as, for example, (i) "*But Jehoash king of Israel replied to Amaziah king of Judah: A thistle in Lebanon sent a message to a cedar in Lebanon, 'Give your daughter to my son in marriage.' Then a wild beast in Lebanon came along and trampled the thistle underfoot"* (2 Chronicles 25:18); (ii) "*It will produce thorns and thistles for you, and you will eat the plants of the field"* (Genesis 3:18); (iii) "*But land that produces thorns and thistles is worthless and is in danger of being cursed. In the end it will be burned"* (Hebrews 6:8).

Columella [58], in the treatise "*De Re rustica"* (1st century BC), mentioned some interesting references about the use of cardoon as a milk coagulant, for example, "*It will be necessary too not to neglect the task of cheese-making, especially in distant parts of the country, where it is not convenient to take milk to the market in pails. Further, if the cheese is made of a thin consistency, it must be sold as quickly as possible while it is still fresh and retains its moisture; if, however, it is of a rich and thick consistency, it bears being kept for a longer period. Cheese should be made of pure milk which is as fresh as possible, for if it is left to stand or mixed with water, it quickly turns sour. It should usually be curdled with rennet obtained from a lamb or a kid, though it can also be coagulated with the flower of the wild thistle or the seeds of the safflower, and equally well with the liquid which flows from a fig-tree if you make an incision in the bark while it is still green*.".

The only treatise on the ancient gastronomy that is known nowadays is "*Artis magiricoe libri X*", also known as "*De Re coquinaria*" written by Apicio in the first century AD [4]. In this treatise some recipes using cardoon can be found in Chapter XIX of Book III [5], as, *for example,* "*CARDUI ("cardoons"): thistles are eaten with a salty dressing, olive oil, and hard-boiled eggs"* (adapted)*; "ALITER CARDUI ("other cardoons"): rind, mint, coriander, finely chopped fennel, pepper, levis and salt water and olive oil"* (adapted)*; "ALITER CARDUOS ELIXOS ("other cooked cardoons"): served with pepper, cumin, sauce and olive oil"* (adapted). In addition, in Spanish manuscripts of fifteenth century [59], it is possible to find a recipe of candied *C. cardunculus* describing that the ideal months for *C. cardunculus* picking are April/May, when the stalks are more tender and sweet. The process of confection begins with stalk cutting, then rinsed in cold clear water for 1 or 2 days, followed by cooking in clear water and ending in a process of soaking in a syrup of sugar and water. Later, in the seventeenth century, the use of *C. cardunculus* is also mentioned in the elaboration of dishes along with butter, onion, pepper, nutmeg, ginger, eggs, among other ingredients, like milk clot (e.g., "*Almojavanas de quajada*"). The first cookbook printed in Portugal, known as *"Art of Cooking divided in two parts"* (from the original *"Arte de Cozinha dividida em duas partes"*) brought up some references of recipes of the banquets to be served during April, with dishes using cardoon such as "*Cardoon curdled with eggs*", "*Bundle of cardoon garnished with the same cardoon*" and "*Italian style cardoon garnished with cream*". Also in

medicine, several references on the use of cardoon can be found in treatises, such as "*Pedacio Dioscorides anazarbeo, Acerca de la materia medicinal y de los venenos mortiferos traduzido de lengua griega en la vulgar castellana & illustrado con claras y substantiales annotationes, y con las figuras de innumeras plantas exquisitas y raras*". This work consists of the translation from Greek to Castilian of the treatise *De Materia Medica*, by Pedanius Dioscorides (first century BC) with the inclusion of personal annotations. In Chapter XIII is included the description of the benefits of cardoon on the stomach, liver, bladder and kidneys, but also in the prevention of the bad odors from the human body.

As in a previous treatise [58], several ways of preparing and consuming cardoon are reported, especially using young and tender plants, like the preparation using wine, salt and pepper. Andres Laguna [60] differentiates the "cardoon" from the "artichoke" referring to the latter on as "lush" for which reason should be placed in the list of foods recommended to the bride and groom, however can be used as milk coagulant. A manuscript about the cheesemaking process in the Alentejo region, reports different ways of preparing *C. cardunculus* aqueous extracts [61]. The author also points out that the consumption of cardoon can lead to food craving, and mentions a method for growing cardoon: "*The truth is that planting them* [the thistles], *covering all them with earth, makes them more white, more tender and tastier, like they were from a different species*" [60]. This description is in line where Priest Isidoro de Barreira [57], who, referring to the biblical meaning of the cardoon, describes: "(…) *that* [torment] *which he suffers* [thistle] *before being suitable for eating*: (…) *when they bind him, and cover him with earth, in which will be mortified to lose its bitterness*." Cardoon crops are present in paintings from the beginning of the seventeenth century by Caravaggio and Juan Sanchez Cótan [50]. Later during the seventeenth century, de Cabreira [62] included the use of "holy thistle" in the treatment of sores in his compendium of medicines for surgery.

2.3. The natural growth cycle of *Cynara cardunculus* L.

C. cardunculus, as other Mediterranean species, is a plant fully adapted to the local climate conditions, where raining is low, irregular and mainly concentrated in autumn/winter. On the other hand, the hot dry summers are not favorable for plant growth, especially if no irrigation is used. The first stage of *C. cardunculus* growing cycle initiates after seeds germinate, usually in the first weeks of autumn. In this stage, two fresh cotyledons arise from the ground, followed by several leaves and, later, a leaf rosette. This rosette usually grows in a slow but steady manner and, the *Cynara* spp. may take all wintertime to early spring at this rosette stage. By late spring, *Cynara* spp. develops a leaf-branched floral scape including several heads. After full blossom and flower fertilization, the fruits ripen and finally the aerial biomass dries up in the summer. When the weather conditions become milder the perennating buds on the basal plant part sprout and a new development cycle starts. This succession of annual growth cycles may last several years [14].

The cultivation as an industrial crop for industrial application of *Cynara* spp. resembles most of the stages of the natural growth reported before, that is, as a perennial field crop in dry farming. As a perennial crop, and with a very deep plant root system, a basal dressing before sowing is recommended. After subsoiling and plowing are recommended, a thorough harrowing should

be followed. The seed germination occurs when the soils water content and the environmental temperature are favorable, normally in the autumn or spring of Mediterranean climates. After this initial growth cycle, the aerial biomass production is usually lower due to the larger development of the root system, but can increase in the next growth cycles, depending on the environmental conditions. In the case of a cold autumn (early frosts), spring sowing may constitute a better option. It is advisable to accomplish the spring sowing as soon as the period of frosts is over. Usually, plants will reach summertime in the rosette stage; after, and due to the high temperatures, some leaves dry up. Later, when the environmental conditions are milder, *Cynara* spp. resumes its vegetative growth, and the size of the leaf rosette becomes bigger.

After, the growing process of the plant can be considered finished in the next summer and *Cynara* spp. can be harvested [14, 26]. Harvest process should be performed after the conclusion of the plants growing cycle,but before seed dispersal. The aerial biomass of *Cynara* spp. should be dry (less than 15% moisture) and seeds must be ripe. The research and development of proper machinery for harvesting *Cynara* spp. plantations is under process aiming for harvesting the whole biomass in one operation.

2.4. Harvest and conservation of *Cynara cardunculus* L. flower

C. cardunculus adult plant can reach a height of 3 m, spread over an area of 1.5 m in diameter [16], and can contain 15 inflorescences, 7 in average [16]. These inflorescences do not open all at the same time because they have different maturation states.

The flower harvest is performed through a cut, and should be done when the inflorescences are mature and open. To obtain high-quality flower with a minimum of impurities, like straw, the pistils should be collected as high as possible on the plant. It is empirically and generally accepted that the more blue-purple the collected material is, the more value it has for traditional cheesemaking [16], concerning at least the milk clotting activity. The harvest of the flower is usually done between the end of June and the beginning of July [51, 63], depending on the year and on the varieties; it is usually performed manually, with a bucket with two hooks where the inflorescence fits. The scissors used by the pickers are long and very sharp, being able to collect all of the flower at once. There are some recent developments regarding mechanical harvesting attempts, but so far, there is no specific device commercially available.

The traditional preservation process for cardoon flower is at room temperature (25–30°C) with air dehydration for about 30–60 days [67]. The purple parts of the cardoon flower (styles and stigmas) are collected along the flowering season and placed to dry at room temperature, protected from sunlight, and with regular turnings of the material to prevent unwanted fermentations and fungi growth [51, 64]. The drying process can decrease the coagulant activity [65]. In spite the fact that average flower milk clotting activity (MCA)/g of dried, and non-dried flower was similar, the authors refer to losses of milk clotting activity varying from 20 to 50% when expressed on dry basis or nitrogen (N) total basis. The traditional drying process, used to preserve the flower throughout the cheese production season, tends to standardize the flower composition and coagulant activity and although carried out at low temperature leads to high losses of flower enzymatic activity as measured by coagulant activity. The evaluation of the amount of these losses, together with the decline in clotting activity during conservation,

was estimated to be about 75% of the potential enzymatic coagulant available in the flower expressed per unit of dry matter [66]. Reducing exposure time to traditional drying conditions may limit such losses, and it is possible to use dehydration at higher temperatures while shortening the drying time, with MCA losses still lower than those with traditional drying.

Martins [66] also studied the effect of cardoon flower drying under different conditions (25–30°C for 7 days, 50°C for 5 days, and 100°C for 5 h). The author concluded that when compared to traditional drying process (25–30°C for 30 days), MCA average was significantly higher drying only for 7 days (MCA in dry matter about 35% higher). MCA of dried flower at 55°C for 5 days in a dry basis, was about 17% higher, while drying at 100°C for 5 h caused a loss for flower MCA of about 5% in a dry basis. The moisture content of the dried flower at 25–30°C for 7 days, about 6% (w/w), was similar to that of traditionally dried flower, showing average water activity (a_w) of 0.585. This means that by controlling the moisture content throughout, the drying period can be decreased, and thus the MCA can be significantly preserved if adequate storage conditions are initially respected. Although there are always losses related to flower storage, the dried flower remains well preserved until next harvest period under conditions of reduced relative humidity and at room temperature. After 300 days of dry flower storage at 25°C, Martins [66] found MCA losses of about 35% of the original MCA, near the MCA losses after storage at 4°C for 150 days.

3. *Cynara cardunculus* L. cheesemaking applications

In most Mediterranean countries, Asia and Africa the milk from small ruminants (goat and sheep) is widely used for human consumption, or mainly processed into typical cheeses by traditional manufacture methods, in contrast to most Anglo-Saxon and Northern European countries, where small ruminants meat production is the only purpose [67]. Although, the amount of milk produced per ewe or goat is extremely variable, depending on the geographical location and prevailing weather conditions, and the farming system, a marginal farm where ewes are milking after weaning the young, in contrast to a dairy farm, where milking occur during whole lactation period [68, 69].

Milk and cheese production from small ruminants in Mediterranean countries has a great socio-economic relevance, mainly in rural areas. In Portugal, according to the latest published statistics [70], sheep milk production reached 68.6×10^6 L/year, and goat milk 25.6×10^6 L/year, and almost all is used for cheesemaking, recording 11,400 and 2400 tons of sheep and goat cheese, respectively.

Specific sensory characteristics of ewe and goat cheeses are related with the chemical composition of raw ewe and goat milk, the coagulant enzymes, raw milk microbiota or milk inoculation with autochthonous strains, and some distinctive manufacturing cheese practices [71]. Traditionally, pistils of wild and cultivated cardoons are used to produce several traditional ewe's and goat 's cheeses, namely Serra da Estrela, Serpa, Nisa, Azeitão and Évora in Portugal [72]; La Serena, Los Pedroches, Torta del Casar, Los Ibores and Flor de Guía in Spain [42, 65, 71, 73–80] and Caciofiore, Fiore sardo, Cacio Fiore and Cacioricotta cheeses in Italy [81]. Some

of these cheeses benefit from protected designation of origin (PDO) status in which *C. cardunculus* flower aqueous extracts have been successfully employed and legally required (**Table 1**).

3.1. *Cynara cardunculus* L. flower aqueous extracts

With the exception of some standardized formulas recently commercially available, the general preparation of cardoon flower extracts for cheesemaking use, remains generally as described in very old references. Coagulating enzymes are extracted from dry flower (styles and stigmas) on a day-to-day basis [83]. The required amount of cardoon flower is placed in bottled water infusion during a variable period of time, being carefully macerated and ground with water, usually in a mortar. The mixture is then filtered, obtaining a purplish or brown liquid which is added to the milk [71, 80].

This traditional use is based on the observation of previous clotting times, and when appropriate, compensation for any loss of enzyme activity coagulant is made by correcting the amount of flower applied per liter of milk as a correction given the empirical use of the coagulant. Control of extracts coagulant activity is performed by controlling the amount of flower necessary to the milk batch volume, frequently equivalent to 0.2–0.6 g flower/L milk [84].

So, the cardoon extract is an aqueous extract of edible parts of flowers from plants, prepared with a variable proportion of bottled water, sometimes with some salt addition during maceration for flower proteinases extraction, therefore usually not standardized. With the maceration in a mortar, with a 5% sodium chloride solution and sand added as an abrasive

Cheese	Country	Type of milk	Coagulant (reference in law)
Mestiço de Tololosa	Portugal	Ewe and goat	Animal Rennet or *Cynara cardunculus*
de Nisa	Portugal	Ewe	*Cynara cardunculus*
de Castelo Branco	Portugal	Ewe	*Cynara cardunculus*
de Évora	Portugal	Ewe	*Cynara cardunculus*
Serpa	Portugal	Ewe	*Cynara cardunculus*
de Azeitão	Portugal	Ewe	*Cynara cardunculus*
Serra da Estrela	Portugal	Ewe	*Cynara cardunculus*
La Serena	Spain	Ewe	*Cynara cardunculus*
Torta del Casar	Spain	Ewe	*Cynara cardunculus*
Flor de Guía	Spain	Ewe, goat and cow	*Cynara cardunculus* or *Cynara scolymus*
Media Flor de Guía	Spain/Gran Canaria Island	Ewe, goat and cow	≥50% *Cynara cardunculus* or *Cynara scolymus* ≤50% animal rennet
Guía	Spain	Ewe, goat and cow	*Cynara cardunculus* or *Cynara scolymus* or animal rennet

Table 1. PDO cheeses made with aqueous extract of ***Cynara cardunculus*** L. in Portugal and Spain [82].

agent, followed by infusion over 2 days under agitation, Christen and Virasoro [85] managed to obtain almost the maximum flower coagulant activity. With a similar extraction process, Tsouli [86] concluded that the sodium chloride concentration of the extracting solution does not influence the enzyme extraction; however, it is necessary to ensure a minimum ionic strength since less active solutions are obtained with water. Perhaps the ionic strength is not so important in this type of extraction, since extraction follow the destruction of the vegetal tissues, although not complete. On the other hand, the results of Tsouli [86] support the use of warm water for extraction. The author obtained more effective extractions at temperatures of 23°C than at 4°C, although very long extractions at higher temperatures may lead to the development of molds; high salt concentrations may have a favorable effect, acting as inhibitors of microbial growth in the extracts.

Regarding more intrusive destructive processes, Sousa and Malcata [87] obtained effective extractions with less than 1 min flower mill time, an extremely destructive process; the optimum pH for extraction was 5.9 (similar to water), being salt concentration and homogenization time not relevant parameters for extraction efficiency. Martins [66] found that traditional extraction using mortar maceration produced extracts with MCA 3 times greater than with ultrasonic extraction, and about 1.5 times higher than with an high speed blender, using the same extracting solution (5% NaCl solution) and same temperature extraction. However, the latter process is more practical for the preparation of large quantities of coagulant and is widely used in dairies, both in Portugal and in Spain.

The purple-brown liquid used for milk coagulation is frequently contaminated with other flower components not relevant for cheesemaking, for example, phenolic compounds, which may affect the enzymatic activity and even hinder any purification and concentration processes [88, 89]. This liquid can be preserved to further use at 4°C, although some losses on MCA where noticed from different authors. Tavaria [9, 90] obtained a decrease in extract coagulant activity (65%), after 4 weeks stored at 4°C, lower than for the same lyophilized extracts (34 and 38%, respectively, as extraction was prepared with water, or citrate buffer at pH 5.4). Although, after 1 week the activity losses were reversed, 23% for fresh extracts and 44 and 61% for the lyophilizates, attributed to a spontaneous loss of the catalytically active conformation over time. The proteolytic activity of lyophilized extracts tended to decrease with shelf life and with lyophilization, mainly due to the lower degradation of alpha-casein and also beta-casein, which led to an increase in the coagulant activity/proteolytic activity ratio. Martins [66] also obtained MCA losses of aqueous extracts, from about 27 to 40%, over a 90 day storage period at 4°C. The results revealed similar losses to those obtained for flower conservation, which indicates the possibility to consider extracts preservation at 4°C, allowing the availability of liquid standard solutions for use in cheesemaking. As with other cheesemaking coagulants, according to the same author, finding suitable formulations for better preservation will enhance the exploitation of the cardoon coagulant and its proteolytic potential.

Obviously, there are small variations which are adaptations designed to meet the latest hygienic requirements, or to solve difficulties in cheese manufacture originated by the evolution of production systems. In fact, some different ways to prepare cardoon aqueous extracts are described [71].

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The traditional method of preparing coagulant extracts from *C. cardunculus L.* dried flower is so far mandatory for the manufacture of some Portuguese cheeses with PDO [84], the same occurring for some spanish PDO cheeses [42], where it is also possible to use powder vegetable coagulant [91]. Standardized solutions of these vegetable proteinases can be obtained in the market for the production of fresh and ripened sheep's milk cheeses in Portugal and Spain, whereas other preparations of proteases from *C. cardunculus* have been recently approved in Canada and accepted in different countries for use in cheesemaking [92].

3.2. *Cynara cardunculus* L. proteases as milk clotting enzymes in cheesemaking

The cardoon extract used in traditional cheesemaking is an aqueous extract of edible parts from *C. cardunculus* L. flower, especially the styles and stigmas. The final crude extract contains a mixture of acid or aspartic proteinases (endopeptidases), the same type of enzymes used for cheesemaking, like chymosin, pepsin and some other proteases from microbial origin. These proteinase combinations allow the milk casein micelles destabilization, and their subsequent coagulation by micellar aggregation using the milk ionic calcium, the most common milk coagulation process for cheesemaking.

Aspartic proteinases are widely distributed in nature, and have been extensively detected and isolated in seeds, leaves and flowers in different plants [93–95] . These enzymes are assigned important functions in animal biological systems, namely protein degradation (pepsin, chymosin and cathepsin D) or blood pressure regulation (renin), among many others, but their biological functions in plants are not yet clear, remaining still as hypotheses [94, 95]. In general, they have been involved in protein processing or degradation in different plant organs, pollen-pistil interaction, as well as in plant senescence, stress responses, programmed cell death and reproduction [94–96].

Several aspartic proteinases have been identified in *C. cardunculus* L. flower extracts, which have been assigned different names, cardosins A–H [41, 96, 97], cyprosins 1–3 [98], cynarase [93, 99], which are generally designated at MEROPS database by Phytepsin, ID A01.020 (Clan AA, Family A1), belonging to the IUBMB group EC 3.4.23, namely EC 3.4.23.40 with the chemical name of phytepsins, including cardosins and cyprosins.

C. cardunculus pistils are known to express several aspartic proteinases; the latest studies bring the total number of aspartic proteinases isolated from pistils of *C. cardunculus* L. to nine, one of the highest numbers of aspartic proteinases isolated from a single organism, suggesting important and specific biological functions within *C. cardunculus* [97]. This multiplicity in pistils is unusual since most plant aspartic proteinases described were mainly isolated from seeds or leaves, at lower concentrations [100]. In this species, the presence of these enzymes is restricted to the pistil, namely in the violet fraction, corresponding to the stigma and stylet, or to the upper part of it [95, 96, 98]. The continuous accumulation of aspartic proteinases increases during flower development, and maturation [41, 98, 99]; Veríssimo et al. [94] reports that the enzymatic complex concentrates mostly within stigmas, and can constitute more than 60% of the total protein of mature stigmas, which, according to the authors, constitutes the first example of extremely high levels of aspartic proteases in higher plants.

These enzymes are characterized, in mature form, by having a tertiary structure with two heterodimeric lobes or domains, two glycosylated subunits with different molecular weight (30 and 15 kDa, variable, depending on the different enzymes which have been identified in the enzyme complex), between which is located a large cleft where the catalytic aspartic centers are located [93–95]. The proteases are inhibited by pepstatin, and shown to be active at acidic pH, with increased proteolytic activity at pH values of about 4.5–5.5 [94, 97, 101–103]. A characteristic feature, of the majority of plant aspartic proteinase precursors, is the presence of an extra segment of about 100 amino acids, known as the plant-specific insert, which is usually removed during processing and is absent from the mature form of the enzyme [95], bearing no sequence similarity with aspartic proteinases of mammalian or microbial origins [94]. As the majority of coagulating enzymes used in cheese manufacture, aspartic proteinases from cardoon flower crude extract reveal primary affinity for breaking the link Phe105-Met106 of κ-casein, an action that triggers enzymatic milk coagulation process, having a subsequent action on the α_s- and β-casein, with preferential affinity for peptide bonds involving hydrophobic amino acids [41, 44, 97].

Aside from the primary role of cardoon flower extract in cheese manufacturing, as milk clotting agent, there are other important actions during draining and pressing steps, and, in particular, throughout the ripening phase, which have impact in the final product characteristics, depending on the technologies and therefore on the cheese type. After milk preparation, cardoon flower extract is added and dispersed homogeneously by stirring, as all other enzyme coagulant type, after which the milk is left to stand for a gel formation. The extract aspartic proteinases have as primary action the cleavage of the κ-casein Phe105-Met106 link, which triggers the destabilization of the milk micellar casein structure [104, 105]. This reaction allows the calcium sensitive α_s- and β-casein to gradually aggregate, forming a progressively structured protein mesh in which the fat, and other components of milk are retained, the curd.

The cheese production proceeds acting differently on the curd to produce different types of cheese. After milk coagulation, the next phase is whey draining, through various operations, which vary with the cheese type to be manufactured. Although most of the added coagulation enzymes are lost through whey draining, as with other aspartic proteases used in cheese production, residual proteinases, added via cardoon flower extract, remain in cheese. This residual fraction plays, however, an important role in defining cheese properties, and typical characteristics through proteolytic action on casein fraction after coagulation. The residual proteinases influence the progress of proteolysis along cheese ripening, which takes place at lower temperatures (8–16°C). Thus, cardoon flower extract plays a complementary role, which is essential for cheese properties being assigned to specific and technologically essential consequences, such as particular textures in milk sheep cheeses, which will be considered on Section 3.4.

With a good milk quality, the endogenous factors do not cause any inactivation of clotting action of the cardoon crude extract proteases. The ideal temperature of the clotting enzymes depends on the technological temperature profile, and not on enzymes temperature sensitivity, since they reveal proteolytic activity within the temperature range normally used in coagulation phase of cheese manufacture (28–36°C). For cardoon flower extracts, the cheesemaking temperature is limited by lower minimum temperature necessary for the micellar aggregation (about 20°C), being the upper limit dependent on the protease inactivation

temperature (60–70°C), substantially higher than the chymosin inactivation temperature [66, 80], allowing higher cheesemaking temperatures as those used in the manufacture of some traditional fresh white Portuguese cheeses.

Vieira de Sá and Barbosa [80, 106], at pH 6.6, report a significant increase in coagulant activity to about 50°C, increasing them slower up to 70°C, where the activity is maximal, followed by a sharp decrease above this temperature and disappearing at 75°C. Christen and Virasoso [85, 107] referred before a maximum of activity to 68°C, well above 41°C for animal rennet, while Campos et al. [108] observed coagulation difficulties at 20°C and a rapid increase in coagulant activity up to a maximum in the range of 40–60°C, from which activity is lost due to protein denaturation. For *C. humilis*, Martinez and Esteban [109] found similar results, with the coagulant activity remaining around 75°C, decreasing quickly thereafter; for the different enzymes studied, this profile was only surpassed by papain, which remained active until the highest temperature used (80°C).

The temperature effect is more crucial during micellar aggregation than in the primary (enzymatic) coagulation phase. While at low temperatures the enzyme phase becomes slower, the micellar aggregation phase hardly occurs at temperatures below 20°C; at a lower temperature, we can perform the primary coagulation phase without causing the milk to coagulate [110, 111]. Using the Optigraph to study the effect of different technological factors, Alves et al. [112] concluded that milk flocculation time, after cardoon flower extract addition, seemed to be more influenced by temperature than rennet, especially near the limits of the temperature range used in milk clotting for cheesemaking (30–36°C) made from enzymatic coagulation, although these differences were smaller in sheep's milk compared with cow's milk coagulation. However, the micellar aggregation rate increased more with increasing temperature with the cardoon extract than with rennet; from 26 to 34°C, even though the impact on the flocculation time is lower. This means that the use of lower coagulation temperatures, below 30°C, does not benefit the manufacturing technology, and may even create difficulties in draining and subsequent problems during ripening by the instability created in the inner cheese ripening conditions [111].

The milk pH, an important milk property depending on its composition and preservation, is one of the factors that most influence the coagulation in its different phases, primary or enzymatic phase, micellar aggregation and gel firming and syneresis [113]. Modification of milk pH before coagulation is often used as a standardization process in the cheese industry but in traditional technologies the milk pH variability, which originates from the milk composition or preservation, can explain much of the milk behavior within coagulation, which usually leads to problems in the syneresis and draining and, later, in cheese ripening; the aforementioned heterogeneity in traditional cheeses may even have its origin in this fact [111].

The lowering of pH tends to decrease coagulation time by approaching the optimum pH for the proteolytic activity of coagulating agents such as aspartic proteinases of rennet, or *C. cardunculus* flower extracts, whereas above pH 7 there is no coagulation due to the rapid inactivation of rennet enzymes [110, 114]. Likewise, the gel is firmer at a lower pH, favoring the micelle aggregation reaction by decreasing the micelle stability, coupled to the negative charge neutralization and the release of calcium ions from the dissolved complexes and the colloidal phase [110, 114, 115].

The decrease in pH accelerates the enzymatic action and the micellar aggregation [111, 112, 116]; the milk with a pH higher than 6.7 is slow to coagulate, and the gel firmness is affected while milk of pH lower than 6.6 show rapid coagulations and the gel firmness is higher and reached faster [111, 112, 117]. Milk that naturally provide weaker curds suffer more with lowering the pH; in goat's milk with very low pH (6.3–6.4) the curd spontaneously breaks and it becomes very difficult to control the characteristics of curd and fresh cheese [111, 118]. When comparing the effect of pH on the rennet and the cardoon flower extract coagulant activity, the main difference in cow's milk is that the former is more effective mainly at lower pH, although the differences between coagulants are less evident for pH values as low as 5.8 [80, 106]. However, with sheep's milk, the cardoon flower extract is more effective than the rennet for all the pH levels studied by these authors, with differences between coagulants much more pronounced than with cow's milk. Similar results were obtained by Martinez and Estebán [109] for extracts of *C. humilis*, which showed that, for the common pH range for sheep's milk, the coagulant action of vegetable coagulant extracts is less affected than the rennet activity. The authors concluded that the coagulants of animal origin are more sensitive to the milk pH variability, and may reveal some difficulties in the coagulation of sheep milk with high pH (6.7–6.8), as it frequently happens.

The micellar aggregation phase of milk enzyme coagulation is a set of reactions dependent on milk composition in terms of protein and mineral elements, in particular calcium in ionic form, that is, a set of reactions not directly dependent on the coagulating enzymes. However, the type of coagulant influences it indirectly through the proteolytic action exerted on the protein destabilized by the coagulant primary action, interfering with the speed and the firmness of the gel. In fact, the coagulants play an important role in the definition of the characteristics of the curds [115], in conjugation with the more intense proteolytic action that is recognized for the enzymes of the *C. cardunculus* flower extracts. In fact, several authors pointed out a significantly higher proteolytic activity/coagulant activity ratio (P/C ratio) for cardoon extract enzymes compared to chymosin, or even rennet, which includes a percentage of pepsin, although this effect is less evident in cow's milk when compared to what happens in ewe's milk [119]. Alves et al. [112] observed that micellar aggregation rate and gel firmness were lower for milk clotted with cardoon flower extract when compared to rennet coagulation and attributed some of this behavior to the characteristic higher non-specific proteolytic activity of proteases of *C. cardunculus* L. flower, since the micellar aggregation phase is not directly influenced by the coagulant enzymes.

The effect of calcium in the enzymatic coagulation for cheesemaking is well known and it is considered an important technological factor; it is essential for micellar aggregation, especially its Ca^{2+} ionic form, whose proportion depends on milk pH [110, 114, 120]. For this reason, calcium chloride addition to cow's milk, is a common practice in the cheesemaking industry, as an attempt to optimize both the cheese yield, and the curd properties [115, 121], assuming that the milk does not provide the required amount of calcium. It is generally considered that this need does not apply to milk from small ruminants [122], but this may not be true nowadays considering the intensification of sheep and goat milk production. In cow's milk coagulation with cardoon flower extract, Alves [116] and Alves et al. [112], using Optigraph, concluded that gel firmness tends to increase with the addition of calcium, albeit on a lower scale than that for the rennet, which can be attributed to the conjugate effect of calcium chloride addition and the higher non-specific proteolytic activity of the cardoon flower enzymes. In accordance with Vieira De Sá and Barbosa [80], the same authors also concluded that calcium chloride

addition decreased milk coagulation time with cardoon extract and rennet, but from a calcium chloride addition of 0.06%, the difference in the coagulation time from both coagulants almost disappeared, as pointed out by Martinez and Estebán [109] for *C. humilis* and pepsin. In fact, since calcium does not participate directly in the primary (enzymatic) coagulation phase, the decrease in coagulation time is due to a decrease in milk pH, which as mentioned, influences coagulation time [115, 123, 124].

Finally, concerning the traditional cheesemaking technologies, the addition of salt to milk must also be considered as an additional technological factor, since it is a practice present in some cases, as in the Portuguese cheeses of Azeitão, Serpa and Serra da Estrela [84], all of them made with cardoon flower extract as the coagulant agent. The addition of sodium chloride causes a decrease in milk pH, resulting in calcium and phosphorus solubilization. As a consequence, the curd tends to form slowly, and the syneresis is considerably inhibited, contributing to a retention of whey in the curd [125–127]. Although favored by a slight increase in ionic strength, the enzymatic reaction is affected by its excessive increase, despite the pH decrease [114], with animal rennet being less affected than some coagulants of microbial origin [128]. In the manufacture of Azeitão cheese the salting in milk is performed by the addition of 15–25 g salt/L, which can decrease the milk pH in 0.2–0.4 units [129]. Alves et al. [112] found that rennet was clearly inhibited by the addition of salt to milk, whereas for cardoon extract coagulation time and gel firmness remained almost unaffected. Cow and goat milks are very sensitive to salting in milk, but sheep milk shows a greater resistance to this effect, starting from a gel firmness characteristically superior, which is based on the content and type of caseins of this milk type [111].

Despite the studies already done and the knowledge available on the properties of the cardoon flower enzymes, the enzymatic content of the extracts and the effect of the flower variability/enzymatic profile is not fully understood, and the use of cardoon flower extracts still remains somewhat empirical, without any kind of standardization or evaluation of coagulant solutions [65]. However, in recent years, there has been some effort toward the availability of aspartic proteinases or extracts from *C. cardunculus* with guaranteed efficiency in the manufacture of cheese. A laboratory in Spain has been selling a coagulant extract of plant origin for a number of years, claimed to be obtained from *C. cardunculus*. Almeida et al. [130, 131] have developed and characterized a new cardosin B-derived coagulant produced in the generally regarded as safe (GRAS) yeast *K. lactis*, which they claim to be effective in the manufacture of cheese from milk of different species. In 2015, a cyprosin was claimed to be authorized for use in Portugal, Spain, France and the Netherlands, and joined the list of authorized food enzymes in Canada, after favorable decision from Health Canada's Food Directorate [92], and since 2015 a number of applications by different entities for the inclusion of cardoon enzymes or extracts in the European list of food enzymes in accordance with Regulation (EC) No 1331/2008 are waiting for official approval.

3.3. Effect of *Cynara cardunculus* L. on physicochemical, texture and sensory cheese properties

Cheese maturation is a dynamic process, in which many metabolites, resulting from primary degradation act as substrate for secondary reactions [132, 133]. Proteolysis, the main biochemical process that occurs during cheese maturation [111, 134], has a central role in cheese texture development [42], and is usually divided in primary and secondary proteolysis.

Proteolysis is translated by the hydrolysis of the Phe105-Met106 binding of k-casein, which leads to the formation of para-κ-casein and glycomacropeptide, causing micellar destabilization, which results in the existence of a more hydrophilic part of k-casein [104, 132, 135]. Most of the glycomacropeptide is eliminated to the whey, but the para-κ-casein remains in the casein micelles and is therefore incorporated into the cheese [132]. The residual coagulant agent, as well as plasmids present in the curd, will act on α- and β-caseins, giving rise to insoluble and water-soluble fractions, which are high and medium molecular weight peptides [74, 135, 136] (primary proteolysis). These are then degraded into small peptides and amino acids [136, 137] by proteases, and from starter or secondary cultures (secondary proteolysis), which will subsequently contribute to cheese flavor formation [43, 74, 136].

Therefore, it seems obvious that the residual cardoon enzyme fraction remaining in cheese after whey draining should play a specific and technologically important role, promoting particular cheese properties, like textures (softening) in milk sheep cheeses [74, 138]. Vasconcelos et al. [129] studied the Azeitão cheese, having established the basis for its definition and certification as a PDO, and found that the cardoon is the main factor of the typicality of this cheese, and based in detailed studies on different sheep microflora and the milk cheeses whose technology include cardoon as coagulant, many authors concluded by the unique properties resulting from the technological use of the vegetable extracts.

The type of coagulant agent is one of the main factors responsible for the variability in cheese characteristics, being therefore its effect on the proteolysis a subject of profound study. In general, differences in coagulant action on protein can affect the concentration of α-casein and β-casein, and influence the concentration of degradation product, γ-caseins and para-α_s-caseins, which in turn contribute for cheese properties. Thus, several studies have been performed in order to evaluate the influence of different preparations, of the amount of *Cynara* aqueous extract, in comparing it with other coagulating agents or the possibility to use *C. cardunculus* enzymes for cheese ripening acceleration.

Roa et al. [73] demonstrated that in La Serene cheeses the residual coagulant of *C. cardunculus* in cheese, and whey are, respectively, 27 and 78%, of the total amount added to milk. In this type of cheese, cardoon flower extract proteinases play an important complementary role for cheese properties; cheeses made with cardoon extract showed a more intense secondary proteolysis than those made with rennet or microbial coagulants, with hydrolysis of α_s- and β-casein up to 82 and 76% higher, respectively.

In Torta del Casar, Delgado et al. [74] indicate, in general, a weak proteolysis at the first 30 days of ripening, more intense between 30 and 60 days, but without differences between 60 and 90 days of ripening. For this type of cheese, the results show a slow degradation of α_{s1}-casein in the first 30 days in contrast with a high degradation level between day 30 and day 60 of ripening. Unlike α_{s1}-casein, β-casein showed the fastest degradation levels in the first 30 days of maturation and after this time it was slight and constant up to the end of ripening. At 90 days of ripening, the degradation of α_{s1}-case was higher than β-casein, which demonstrated a lower proteolysis level, reaching 38% of the initial level at 90 days maturation. For Serena cheeses, Roa et al. [73] reported a similar proteolysis pattern along ripening period, with a higher proteolysis level of β-casein than for α-casein during the first 30 days of ripening

(percentage of degradation of β1-, β2- and α_{s1}-casein was 41, 58 and 9%, respectively). This rate of casein matrix is associated to cheese texture variation along maturation, decreasing the hardness and consistency of cheeses and increasing their adhesiveness [73].

The proteolysis pattern in cardoon flower cheeses seems to confirm the suggestions that proteinases from *C. cardunculus* displayed a stronger preference for peptide bonds between bulky hydrophobic amino acid residues than chymosin [44, 104]. The use of raw ewe milk and plant coagulant provides these cheeses a spreadable texture, and a peculiar slight bitter taste. The slight bitter flavor of Torta del Casar cheese may be due to certain sequences in the caseins which are particularly hydrophobic and, when excised by proteinases can lead to bitterness [139]. Nevertheless, others studies show that, while α-casein decreased throughout ripening, β-casein only decreased slightly, confirming its greater resistance to hydrolysis [140].

A number of studies tried to evaluate differences between the utilization of animal and vegetable coagulants or to investigate the possibility of reciprocal substitution of rennet by cardoon extracts, concerning the effect of different proteolytic pattern of the different enzymatic complexes and the effect on cheese properties.

For Los Pedroches cheese manufactured with both animal and vegetable coagulating agents, Férnandez-Salguero and Sanjuán [141] demonstrated a decrease in the relative proportion of α_s-caseins during the maturation in cheeses. The initial proportion of α_s-caseins was higher for the vegetable coagulant (42.3%), and their decrease was also faster during the maturation period. β-Caseins showed a slight decrease in their proportion during cheeses maturation with a higher residual protein content in cheeses produced with animal rennet. The content of compounds located in the γ-casein region were similar for the two coagulants types, increasing from 14.6% (at the beginning of maturation) to 24–25% at the end. These compounds are the result of the proteolytic action of animal or vegetable coagulant agents on β-caseins.

Freitas and Malcata [79] in Picante da Beira Baixa cheese, from Portugal, concluded that coagulation with vegetable coagulant results in more extensive degradation of α-casein, comparatively to animal rennet. β-casein shows a greater resistance to the coagulant agent enzymatic activity, when compared with α-casein. The water-soluble nitrogen for cheeses coagulated with animal rennet were in general lower than those for cheeses coagulated with plant rennet, but much minor differences were identified on non-protein nitrogen. These results are in agreement with those presented by Marcos et al. [142] which reported a small degradation of β-casein in several Portuguese sheep, goat and bovine milk cheeses.

For Serpa cheese, Roseiro [71] compared the effect of replacing *C. cardunculus* by animal or microbial rennet as coagulant. The pH 4.4 soluble nitrogen (pH 4.4-SN) was significantly higher for cheeses made with *C. cardunculus*, while trichloroacetic acid soluble nitrogen (TCA-SN) and phosphotungstic acid soluble nitrogen (PTA-SN) were significantly higher for cheeses made with animal, or microbial rennet. However, at 60 days of ripening no significant differences were observed. The proteolysis of α_{s1}-casein was faster than the one of β-casein, with α_{s1}-casein being completely degraded at the end of maturation. After 90 days of maturation β-casein showed a lower proteolysis level, but a faster degradation, when compared to α_{s1}-casein during the first 30 days period; up to the end of ripening, the levels of β-casein

remained practically constant. The electropherograms of Serpa cheese caseins, obtained through capillary zone electrophoresis (CZE), showed a peak that remained throughout maturation, and was not detected in others cheeses. This peak was suggested as arising from β-casein and allows the identification of the coagulant type employed in the cheesemaking [71]. Alvarenga et al. [143] concluded that the softening of Serpa cheese occurs during the first 2–3 weeks, and it can be explained by the breakage of the protein-protein bonds, which occurred during proteolysis, and to the dissociation of sub-micelles caused by a pH decrease.

O'Mahony et al. [144] studied the effect of type and amounts of coagulant (100% *C. cardunculus*, 50% *C. cardunculus*/50% chymosin, 25% *C. cardunculus* and 75% *C. cardunculus*, Chymosin, 100%) in miniature Cheddar-type cheeses. According to the results, there were no substantial differences between the compositions of cheeses made using any of the four coagulant mixtures. Otherwise cheeses manufactured with coagulant mixtures containing *C. cardunculus* proteinases showed higher levels of pH 4.6-soluble nitrogen, and higher degradation of α_{s1}-casein, than cheese made with chymosin as coagulant.

C. cardunculus proteinases, incorporated in coagulant mixtures, has the possibility of accelerating proteolysis of Cheddar cheese. Tejada et al. [77] on Murcia al Vino (goat's cheese) observed an intense proteolytic activity during the maturation process, particularly during the first 30 days, when *C. cardunculus* aqueous extract were used. At 15 days of maturation the levels of α_s-caseins and β-caseins were significantly lower in cheeses produced with vegetable coagulant, meaning that α_s-casein degradation during maturation was more intense than in the cheeses produced with the animal origin coagulant (59.5 and 40.2%, respectively). However, the degradation of β-casein was very similar in both cases. The γ-CN concentration was significantly higher in cheeses manufactured with animal rennet as cyprosins in the vegetable rennet cheeses hydrolyse such fractions or maybe inhibit plasmin action. Similar results were observed by Pino et al. [43] in goat's cheese made with animal coagulant and with powdered vegetable coagulant. Significant differences in the remaining percentage of β-caseins from day 2 onwards were not observed, with this remaining practically constant until 120 days of maturation. However, α_s-caseins levels decreased between day 2 and day 15, for both types of coagulants, decreasing slightly thereafter and more intensely in cheese made with *C. cardunculus*.

In Évora cheese [145, 146] the proteolysis is not very pronounced in contrast to the high lipolysis, resulting mainly in secondary metabolites compounds, such as amino acids, products of amino acid catabolism and mainly free fatty acids and other volatile compounds (esters, ketones, aldehydes, alcohols, lactones, among others) with consequences on the sensorial characteristics of cheese The differences between cheeses manufactured with *C. cardunculus* or with animal rennet is more pronounced in the early stages of maturation (30 days vs. 45 and 60 days) and were explained by the different peptide profile, the greater increase of amino acids (40 vs. 8%) and higher intensity of ewe flavor and "typical flavor" supported biochemically by the free fatty acids profile (particularly short and medium-chain-fatty acids) and components such as 3-methyl propanoic, 2-methyl butanoic and 3-methy butanoic (products of amino acid catabolism). The formation of hydrophobic peptides and the ratio of hydrophobic/hydrophilic peptides throughout the ripening are higher in cheeses made with *C. cardunculus* than in those made with animal rennet.

Galán et al. [147] investigated the effect of different amounts of *C. cardunculus* coagulant (normal and the double amount) and calf rennet in sheep milk cheese. After 2 days of ripening, significantly higher levels of cheese casein hydrolysis was achieved (measured as SN-soluble nitrogen, NPN-non-protein nitrogen in cheeses produced with double amount of *Cynara* compared with those made with normal amount and animal rennet. There were significant differences between the SN values of the cheeses clotted with two types of coagulants. In the early stages of ripening, the observed high SN levels were caused by the intense proteolytic enzymes action of vegetable coagulant. The taste intensity of the cheese produced with vegetable coagulant was higher than the one produced with calf rennet except for day 180. Generally, the cheeses made with double *C. cardunculus* were more bitter and acidic than cheeses made with calf rennet but no constant significant differences were observed. Later on, in 2012, Galan et al. [138] tested three coagulants (100% *C. cardunculus*, Calf rennet and 50:50 mixture). For most of the chemical and microbiological parameters no differences were observed between the coagulants used but the proteolysis index was higher in cheeses made with vegetal rennet and mixture cheeses which acquired the typical sensory characteristics faster than animal rennet cheeses.

In the last decade, special attention has also been paid to the effect of cardoon flower enzyme composition on cheese properties, following the hypothesis that the diversity of the thistle flower enzymatic profile may influence the cheese characteristics, since it is possible to differentiate at least the intensity of the proteolysis in the cheese manufacture and ripening.

In 2013, Ordiales et al. [140] analyzed the influence of rennet from different *C. cardunculus* ecotypes plants, selected for its clotting and proteolytic activity on caseins, on the characteristics of manufactured 'Torta del Casar' cheeses. The rennet with higher clotting activity after 24 h of maceration allowed higher creaminess, viscosity, and overall acceptability of the cheese. Nevertheless, the rennet with high proteolytic activity negatively influenced the acidity, bitterness and creaminess parameters. Consequently, it was concluded that the most appropriate cardoons for making this cheese are those with higher clotting activities and moderate proteolytic activities especially on β-casein. Concerning to the relationship between the characteristics of the rennet and the sensorial analysis, the degradation of β-casein was positively correlated with the compactness of the cheese paste, while is negatively correlated with the creaminess and the bitter and acid taste (the greater degradation the less the creaminess, and less bitter taste).

Recently, Guiné et al. [148] evaluated the physicochemical and sensorial properties of the Portuguese cheese "Serra da Estrela" made with six different ecotypes of cardoon flower extract. The results confirmed that the type used rennet, and in particular the cardoon flower ecotype, greatly influenced the cheese properties. A great variability in the chemical composition was verified. Texture characteristics also diverged importantly among samples and color parameters also revealed noticeable differences. The sensorial analysis allowed to clearly identify some differences, particularly in terms of creaminess, rind thickness and uniformity. In a similar studied carried out by Correia et al. [149], cheeses were also manufactured with extracts of different cardoon flower. The results showed that cardoon ecotype had a considerable influence within clotting time, and color parameters. The ecotype that provided the lowest clotting time was also the one with the highest concentration of total cardosins.

This confirms cardosins relevance in clotting time reduction. Cheeses produced with the different cardoons ecotypes were significantly different concerning rind and paste properties, as well as for global sensorial grade.

4. *Cynara cardunculus* L. other traditional and industrial applications: the biopharmaceutical potential

Traditionally, infusions of artichoke and wild cardoon leaves have been used since the fourth century B.C. [150], based on well accepted health benefits, regarding liver protection [151] and stimulating bile flow from the gallbladder (choleretic action) [150, 152, 153]. Artichoke leaves and seed extracts are also consumed to protect toward atherosclerosis, arterial hypertension and hyperuricemia [11, 154]. Wild cardoon leaves are popular in folk medicine, given to their cardiotonic, antihemorrhodial, and antidiabetic actions [155] mainly due to the biological effects of the secondary compounds. Among the different *C. cardunculus* physiological comparts, leaves appeared to accumulate a wide range of compounds with known biological activities [31, 36, 37, 156].

In order to pull out compounds of interest from *C. cardunculus* leaves, extraction processes must be done and optimized. Divided in conventional and non-conventional, extraction methodologies applied to *C. cardunculus* are commonly conventional, as batch and Soxhlet extraction [31, 36, 37, 156, 157], while application of non-convention extraction methodologies, as ultrasound, microwave, supercritic fluid or ionic liquid solutions, are still very scarce [158].

With interesting biological activities, the study of cynaropicrin extraction, a sesquiterpene lactone, found for the first time by Ramos et al. [31] in *C. cardunculus* leaves with a Soxhlet extraction, is an important step for the recovery of this added value compound. Some studies are recently appearing, applying new and non-conventional methodologies for cynaropicrin extraction from *C. cardunculus* leaves, such as ultrasound assisted extraction, where an increase of 36% on cynaropicrin concentration was achieved as well a reduction on energetic costs [159, 160]. *C. cardunculus* biological potential is tremendous, but the challenge is to transfer this knowledge to industry, toward new value chains, being crucial the cost reduction of extraction/purification processes ensuring safety, and end products functionality.

The lignocellulosic fraction, especially of cultivated cardoon, over the years has demonstrated a great potential as solid biofuel. The first research on *C. cardunculus* potential as an energy crop was carried out in the 1980s [161]. Currently research within energy and cardoon is wide, with several reports which highlight different possibilities: solid biofuel [14, 162], liquid biofuel (seed oil [29], biodiesel [163] and bioethanol [22, 164, 165]), and biogas production [20, 24]. Clearly, within energy production, *C. cardunculus* represents a high potential as an alternative to fossil materials.

5. New perspectives for economic valorization

Portugal has applied recently for the registration of traditional *C. cardunculus* crude extract as an enzymatic extract for cheesemaking, which will enable the traditional utilization of cardoon

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as coagulant for cheesemaking to legally proceed. However, deeper knowledge concerning genetic variability within the plant, and enzymatic profiles is critical to improve traditional cheese quality, allowing to find a basis for certified and guaranteed enzymatic formulas for commercial use in cheesemaking, and to reinforce and valorise all the innovative potential associated to *C. cardunculus* use. In the near future, regarding the cardoon as extractives source, the higher demand is searching for cleaner and more sustainable processes, capable of combining bioactivies extraction efficiency, with reduced costs and great biological action, potentiating new biopharma-products development. The fulfill comprehension of the genetic natural variability of *C. cardunculus* plants will be out breaking in terms of full capability to economic crop valorization.

6. Concluding remarks

In agro-industries, there is an increasing interest in promoting integrated exploitation of different biomass resources, in order to maximize crop value. Consequently, a global socio-economic and environmental impact of these industries is expected in the future.

Cynara cardunculus biochemical profile unveils a great potential for different applications within energy generation, as well as in the food and pharmaceutical industries. In parallel, the traditional applications will be maintained, such as the use of *C. cardunculus* flower aqueous extracts for cheese production. For a number of cheeses regulated by PDO in the Iberian Peninsula, the use of this vegetable coagulant is mandatory. The increasing use of vegetable proteases as a coagulating agent is related not only to the unique sensory characteristics of the resulting cheese but also with a growing consumer interest in reducing the consumption of animal-derived products.

Due to the high variability of biochemical profiles of *C. cardunculus*, the development of a basis for certified and guaranteed enzymatic formulas for use in cheese manufacture is mandatory to develop the full innovation potential in this agro-industrial sector. Overall, the assessment of the genetic, chemical and biological diversity of natural occurring populations of *C. cardunculus* will also promote a wider economic valorization, adding new biotechnological applications to the traditional activities. In this way, the consolidation of the knowledge transfer from the perspective of maximizing the exploitation of a value chain around cardoon production will be achieved.

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Conflict of interest

The authors confirm that this article content has no conflict of interest.

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