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Characterization of *Cynara cardunculus* L. flower from Alentejo as a coagulant agent for cheesemaking

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ABSTRACT

The cardoon (*Cynara cardunculus* L.) is a mandatory vegetable coagulant for certain Protected Designation of Origin Portuguese cheeses. It grows wild in Portugal and is used without any type of control regarding flower picking or extract preparation, representing some uncertainty in cheese manufacture. The variability in technological properties, in the context of traditional cheese manufacture, of cardoon flower ecotypes from the Alentejo region was evaluated, including milk clotting and proteolytic activities, coagulation properties and potential cheesemaking yield of flower extracts. Multivariate statistics highlighted the variability of flower properties for cheesemaking, but allowed the aggregation of the ecotypes into five groups under the major influence of milk clotting activity and effect on gel firmness and micellar aggregation rate, followed by proteolytic activity. These differences may have an impact on cheese properties and therefore can allow the selection of cardoon flower for the manufacture of different types of cheese.

1. Introduction

Milk coagulation is the essential step in cheesemaking production process, for which preparations of proteases have been used for thousands of years (Shah, Mir, & Paray, 2014). Coagulants play an important role in the definition of curd characteristics (Ramet & Weber, 1980) by influencing micellar aggregation and interfering with the speed of the gel firming and the final gel firmness. In turn, these effects may influence the curd draining properties, the cheese moisture content and the cheese texture and flavour (Mazorra-Manzana et al., 2013). Calf rennet is the enzyme preparation most used in milk coagulation, but the limited supply and high prices for this coagulant led to search for rennet substitutes over the last fifty years (Shah et al., 2014).

The type of protease (e.g., cysteine, serine and aspartic) and its specificity is of great relevance and defines its use in food processing. For cheesemaking, in general, a protease with a higher ratio between specific activity for milk coagulation (milk clotting activity, MCA) and enzymatic non-specific proteolytic activity (PA) (MCA/PA ratio) is more capable to form curd, obtaining higher yield and less bitterness development during cheese processing, while a low ratio may result in lower curd recovery, weak curd firmness and the release of bitter peptides that affect the sensory properties of the final product (Amira, Besbes, Hamadi, & Blecker, 2017; Mazorra-Manzano et al., 2013).

Unlike the properties of various enzymes of plant origin tentatively used as calf rennet substitutes, cardosins from cardoon flower show suitability for cheesemaking (Roseiro, Barbosa, Ames, & Wilbey, 2003), although several authors

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pointed out a significantly lower MCA/PA ratio for cardoon extract enzymes compared with chymosin, or even rennet, which includes a percentage of pepsin. This effect is less evident in cows' milk when compared with ewes' milk (Cordeiro, Jacob, Puhan, Pais, & Brodelius, 1992).

Cardoon (*Cynara cardunculus* L.) is a thistle variety, which mainly grows in dry and stony areas of Portugal and in some other parts of the Iberian Peninsula (Sales-Gomes & Lima-Costa, 2008). The flower pistils have been used in some regions of the Mediterranean, West African and European countries as a natural rennet substitute to produce traditional sheep cheeses (Cavalli, Lufrano, Colombo, & Priolo, 2013; Fernández Curt, & Aguado, 2006; Roseiro et al., 2003).

Spain and Portugal have the largest variety and production of cheeses using this vegetable coagulant, which are normally produced on an artisanal scale. Nowadays, sheep cheese produced with this type of coagulant has become highly valued because of its special taste and organoleptic properties (Fernández et al., 2006; Roseiro et al., 2003). These vegetable coagulants have other interesting uses concerning specific targets, for example, cheese production with these natural enzymes can be aimed at lacto-vegetarian consumers and ecological markets (Galán, Cabezas, & Fernández-Salguero, 2012; Khaldi, Sonnante, & El Gazzah, 2012) or to overcome religious restrictions (Amira et al., 2017; Roseiro et al., 2003).

The use of the cardoon, *C. cardunculus* L., as a coagulant for cheesemaking is mandatory in certain Portuguese cheeses benefiting from the status of Protected Designation of Origin (PDO) with EU recognition (EC, 1996). However, the plant still continues to grow spontaneously and, in general, flower picking is not controlled, therefore representing some uncertainty in the cheese manufacture. The effect of flower/enzyme profile variability remains somehow unclear and cardoon extract utilisation continues to be made in a traditional way, without any kind of certification or evaluation of crude extract solutions (Martins, Vasconcelos, & Sousa, 1996).

When a potential rennet substitute is studied, it is particularly important to evaluate its milk clotting activity (Fox, 1989; Shah et al., 2014), which refers to the capability of the enzyme for specific κ-casein hydrolysis (Amira, 2017; Jacob, Jaros, & Rohm, 2011). For cardoon extracts these biochemical activities are attributed to the presence of cardosins, whose richness in inflorescence pistils have potentiated its wide use in cheesemaking for centuries (Cavalli et al., 2013; Dubeuf, Morales, & Genis, 2010; Faro, Verissimo, Lin, Tang, & Pires, 1995). Cardoon flower enzymatic extracts have been assigned specific and technologic consequences, mostly in sheep cheeses, as an effect of a more intense PA. In the cheesemaking process this is revealed by quantitative and qualitative differences on α_S- and β-casein proteolysis when compared with the use of other coagulants, with impact on the cheeses biochemical, textural and sensorial properties (Nuñez, Del Pozo, Rodríguez-Marin, Gaya, & Medina, 1991; Pino, Prados, Galán, McSweeney, & Fernández-Salguero, 2009).

In the last decade, special attention has been paid to the effect of the cardoon flower enzyme composition on cheese properties, following the hypothesis that the enzymatic diversity related to the plants ecotype may influence the cheeses characteristics. Ordiales et al. (2013) studied the influence of cardoon ecotype on texture properties of Torta del Casar cheese and also on the overall cheese acceptability. Correia, Vítor, Tenreiro, and Guiné (2016) reported similar influence of cardoon ecotype on Serra da Estrela cheese properties.

Recently, Barracosa, Oliveira, Barros, and Pires (2018a) studied the biodiversity of cardoon from the Serra da Estrela region (Portugal), which is one of the most popular cheese regions in the country. Their research was based on thirty-four morphological characteristics, where the existence of a wide genetic diversity was confirmed. In another paper (Barracosa, Rosa, Barros, & Pires, 2018b), the same group characterised six different cardoon flower cardosin profiles, displaying a wide variation on total and specific cardosin concentrations, important properties for the flower use in cheesemaking. The authors could not find a direct relationship between flower cardosin profile and morphological plant characteristics that was claimed to be relevant for flower productivity and the ease of harvesting.

In this work we surveyed the cheesemaking properties of flowers from cardoon ecotypes of the Alentejo region, in Portugal, using a set of technological properties including the effect on cheesemaking yield estimation and milk coagulation properties in addition to the milk clotting and proteolytic activities evaluation.

2. Materials and methods

2.1. Plant material

Samples used in this study were pistils of cardoon (*C. cardunculus* L.) flowers from 15 ecotypes (Table 1) dispersed throughout the Alentejo region in Portugal, collected along one flowering season, which were coded with the letter 'E', meaning: ecotype; and a number referring their geographic location on the map, as shown in Supplementary material Fig. S1, with no specific order associated. The samples without spines have the suffix 'ns' (no spines) linked to their location number.

The upper purple coloured parts of the cardoon flowers, namely pistils, were cut and separated from the rest of the plant and then carefully picked out to remove waste. All fresh flowers collected were stored immediately for drying for approximately 30 d under room temperature (20-25 °C) and protected from sunlight.

2.2. Preparation of C. cardunculus L. coagulant extracts

The coagulant extracts were prepared from the flower sample of each ecotype following a traditional method preparation for cheesemaking, using 2 g of flower pistils macerated by hand in a mortar, with acetate buffer pH 5.5, and then filtrated through a Whatman No.40 filter adding up to a final volume of 50 mL. The extraction was made at room temperature and further storage was 2–3 °C until extract evaluation.

2.3. Flower extract evaluation assays

2.3.1. Milk clotting activity

The extract milk clotting activity (MCA) was evaluated according to ISO 23058/IDF 199 (ISO/IDF, 2006), using a standard low heat, skim spray dried milk powder as substrate (Actalia Cecalait, Poligny, France), reconstituted to 11% with 0.5 g L^{-1} of $CaCl_2$ solution (Actalia Cecalait) with a final pH of 6.5. For MCA calculations a calf rennet reference standard powder (Chr. Hansen, Hørsholm, Denmark) was used. All assays were accomplished in duplicate.

2.3.2. Monitoring of enzymatic coagulation

The enzymatic coagulation properties were assessed with an Optigraph (Alliance, Frépillon, France), which is based on the measurement of near infrared signal attenuation caused by micellar aggregation (Kübarsepp, Henno, Kärt, & Tupasela, 2005). The Optigraph makes the calculation in real time of all parameters required for the coagulation process: coagulation time, firmness evolution, optimum curd cutting time and organization speed (Optigraph User's Manual). After setting the milk to equilibrate at clotting temperature (32 °C), 1 mL of *C. cardunculus* coagulant extract solution diluted 1:4 was added to each 10 mL of milk. Before

each trial, the intensity of the emitted signal was set to 7 V (Alves, Martins, Mourato, Vasconcelos, & Fontes, 2004). All tests ran for 120 min and the studied parameters were: R (s, clotting time); 0K20 (s, speed of micellar aggregation measured as time to reach a standard clot firmness) and A_{20}, A_{40}, AR and A2R (V, firmness measures, after 20 or 40 min from the beginning of trial or after two or three times R, respectively) (Mahaut, Jeantet, & Brulé, 2000). For the substrate preparation, a non-fat dried milk (Molico, Nestlé, Linda-a-Velha, Portugal) reconstituted to 11% with 0.01 M $CaCl_2$ solution (Merck, S.A., Lisbon, Portugal) was used, pH was corrected to 6.6, with 0.1 M NaOH (Merck, S.A.); the same substrate preparation procedure was used for proteolytic activity and potential cheesemaking yield essays. All analyses were performed in duplicate.

2.3.3. Proteolytic activity

The flower extract PA was assessed by a 12% trichloroacetic acid (TCA) (Sigma Aldrich, Merck, S.A., Lisbon, Portugal) soluble nitrogen (NPN) release from milk coagulation trials at 32 °C for 1 and 2 h. To a 40 mL milk portion (substrate preparation as previously described), 1 mL of *C. cardunculus* coagulant extract solution diluted to 1:4 was added and after the respective action time, the NPN of the TCA 12% soluble fraction was evaluated according to ISO 8968-4/IDF 20-4 (ISO/IDF, 2001), with a Tecator system (Foss, Hillerød, Denmark).

2.3.4. Potential cheesemaking yield

The effect of the cardoon enzymatic variability on potential cheesemaking yield was evaluated from the curd syneresis properties at lab scale coagulation trials according to Remeuf, Leloir, and Duby (1989) with little modification (Martins et al., 2009), consisting on trials of 20 mL of milk. After temperature stabilisation (32 °C), 1 mL of *C. cardunculus* coagulant extract solution was added. Following homogenisation, the milk was left at clotting temperature for 1 h in a centrifuge tube (Ø 28.5 mm) and further centrifuged (3500×*g*, 15 min; Sigma 4K10, Darmstadt, Germany) following a cross-type curd cutting with two orthogonal diameters. After centrifugation the whey volume was measured and the cheesemaking yield on a fresh or dry weight basis were evaluated; the clot dry weight was evaluated according to ISO 5534/IDF 4 (ISO/IDF, 2004).

2.4. Statistical analysis

All experiments were performed in duplicate and the data are the means of two determinations. Statistical treatment was assessed using the Statistica® package for multivariate analysis, principal component analysis (PCA) and hierarchical cluster analysis (HCA, single linkage method).

3. Results and discussion

Table 1 shows the set of average values of 13 technological characteristics evaluated among coagulant extracts of 15 C. *cardunculus* L. ecotypes, showing great variability between them. The results for MCA, a measure of clotting enzymes concentration and a major parameter for cheesemaking, showed a marked variability (57–128 IUMC g^{-1}); only four ecotypes (E1, E2, E3 and E7) showed MCA values similar to the average reported by Martins et al. (1996) for dried cardoon flower (64 RU g^{-1}, approximately 110 IUMC g^{-1}), although MCA of half of the ecotypes were within the ranged referred by the authors (49–78 RU g^{-1}, equivalent to approximately 85–135 IUMC g^{-1}).

However, the results for coagulation time R obtained from coagulation monitoring trials did not reproduce the pattern of MCA for the ecotypes, probably reflecting the effect of the technological parameters (milk pH, coagulant dilution) or differences on the flowers enzymatic profile. Examples of the different patterns referred, can be seen in Table 1 for ecotypes E5, E6, E9, E12 and E15, where fast coagulation was obtained despite the low values for MCA. As for the remaining variables that characterized the coagulation process, such as the evolution of curd firmness, there was a clear and expected dependence on the rate of micellar aggregation. The ecotypes yielding faster micellar aggregation (lower values of 0K20 for ecotypes E3, E5, E9, E9ns, E12, E15) produced firmer curds (A40 or higher A2R).

Again, for PA properties and cheesemaking yield estimate, no clear pattern was identified in relation to milk clotting activity or to the coagulation evolution. The higher PA was found for ecotypes E5, E6, E10, E11 and E12, while the best estimates for cheese yield was obtained ecotypes E7, E9, E9ns, E13 and E15.

In general, the ecotype characterisation showed great variability of flower properties concerning the use in cheesemaking.

Table 1
Averages for 13 technological characteristics evaluated among flowers from 15 ecotypes (E) of *C. cardunculus* L dispersed throughout the Alentejo region.[a]

Ecotype code	MCA (IMCU g^{-1})	R (s)	AR (V)	A2R (V)	A_{20} (V)	A_{40} (V)	0K20 (s)	PA (g NNP 100 g^{-1} Nt)		ΔNNP (%)		Yield (g 100 g^{-1})	Curd DW (g 100 g^{-1})
								1 h	2 h	1 h	2 h		
E1	128	811	1.83	2.78	1.03	2.75	1352	4.72	3.63	121	92	22.8	4.5
E2	126	818	1.93	2.94	1.07	2.89	1250	4.10	3.84	105	97	24.0	4.7
E3	105	901	2.54	3.88	1.09	3.49	968	4.67	3.83	120	97	23.5	4.6
E4	98	916	1.88	2.85	0.78	2.56	1513	4.22	4.74	108	120	26.3	4.8
E5	82	756	2.22	3.43	1.51	3.61	903	5.12	5.07	132	130	23.5	4.6
E6	67	750	1.84	2.81	1.28	2.97	1227	5.02	4.96	129	127	23.9	4.8
E7	105	951	2.08	3.14	0.78	2.72	1274	4.90	4.16	126	105	26.6	4.9
E9	74	779	2.89	4.47	1.82	4.57	640	4.65	5.04	119	129	26.1	5.0
E9ns	89	961	3.02	4.65	0.99	3.91	739	4.46	4.41	114	111	27.4	4.9
E10	58	1177	2.32	3.55	0.12	2.39	1314	5.28	4.44	135	112	26.4	4.8
E11	57	1207	2.09	3.14	0.00	2.07	1619	5.17	4.68	133	118	26.7	4.8
E12	69	734	2.07	3.11	1.51	3.31	997	5.12	4.92	132	126	24.7	4.8
E13	82	692	1.67	2.47	1.35	2.81	1417	4.62	4.44	119	114	28.2	4.9
E14	63	1150	2.20	3.34	0.19	2.33	1415	4.40	4.42	113	112	27.1	4.7
E15	74	823	2.84	4.30	1.59	4.21	683	4.25	4.92	109	126	28.9	5.2

[a] Abbreviations are: MCA, milk clotting activity; R, coagulation time; 0K20, speed of micellar aggregation, measured as time to reach a standard clot firmness; A_{20} and A_{40}: curd firmness at 20 min and 40 min, respectively, after coagulant addition; AR and A2R: curd firmness after two and three times the coagulation time (R) after coagulant addition, respectively; PA, proteolytic activity at 1 h or 2 h after coagulant addition; ΔNNP: non-protein nitrogen increase after 1 h or 2 h of coagulant addition; Yield: yield expressed as g curd 100 g^{-1} milk; Curd DW: Yield expressed as g curd dry weight 100 g^{-1} milk; ns, no spines.

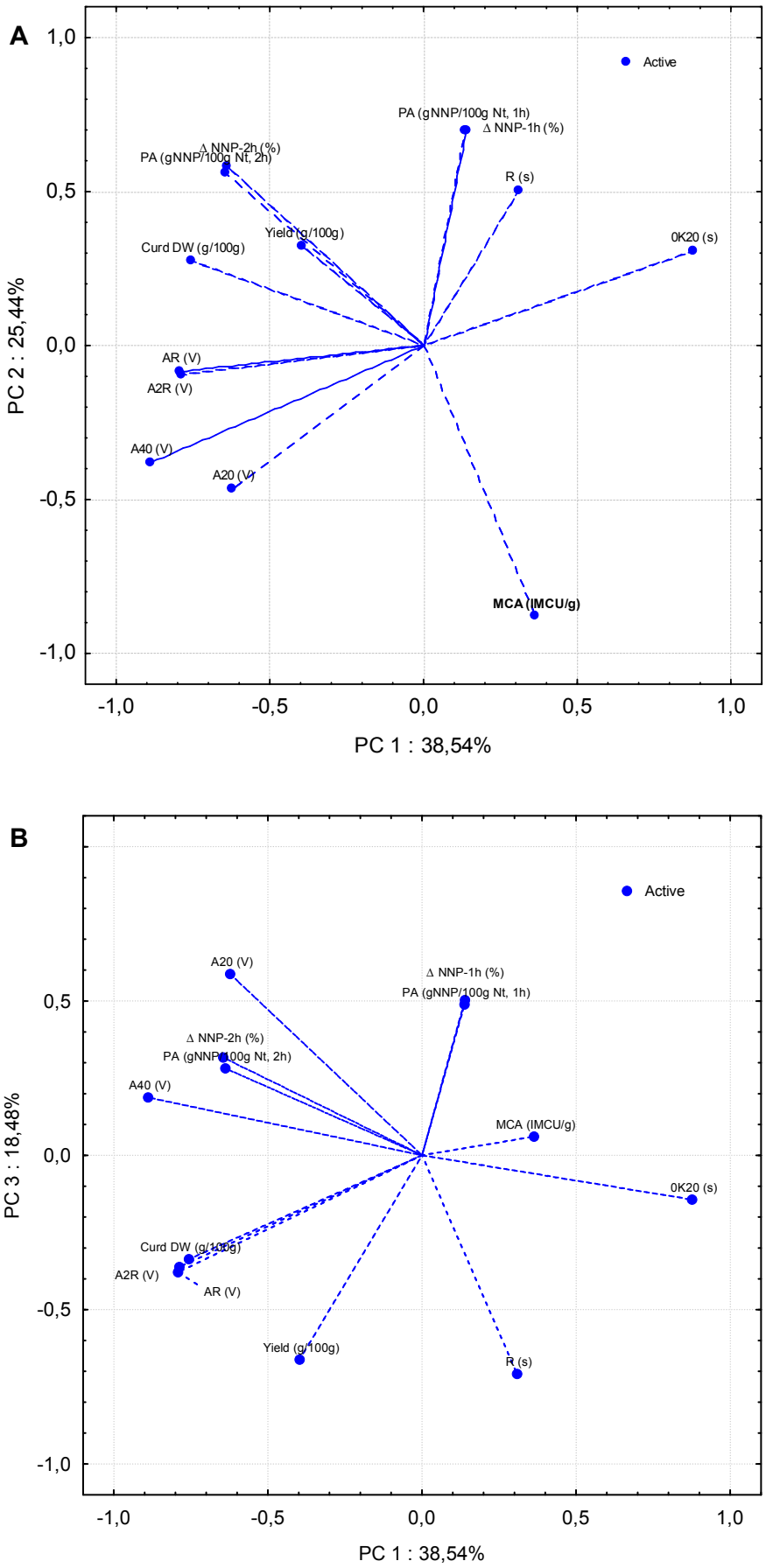

Fig. 1. Projection of 13 technological characteristics evaluated among the 15 *C. cardunculus* L. ecotypes in the plan defined by (A) the two first principal components (PC1 and PC2) and (B) the first and the third principal components (PC1 and PC3). Abbreviations for technological characteristics evaluated are as defined for Table 1.

To integrate all the results for ecotype characterisation and to study the relationship between ecotypes and the technological properties, the dataset was submitted to multivariate exploratory technics. The principal component analysis (PCA) showed that the first three dimensions of the model were found to be significant and explained 82% of the total variance (Fig. 1). The first principal component (PC1) accounting for 38% of the total variation was dominated positively by MCA and 0K20 characteristics and negatively by the curd properties. The second principal component (PC2) accounted for 25% of the total variation and was positively dominated by PA and negatively by MCA (Fig. 1A), therefore opposing the clotting activity to the proteolytic activity. The third principal component accounted for ca. 18% of total variance and is also dominated by the proteolytic and in opposition to the yield and clotting time R.

The first two principal components (PC1 and PC2), accounting for about 64% of the total variability among *C. cardunculus* L. ecotypes (Fig. 1A), generate the ecotype distribution of Fig. 2A where

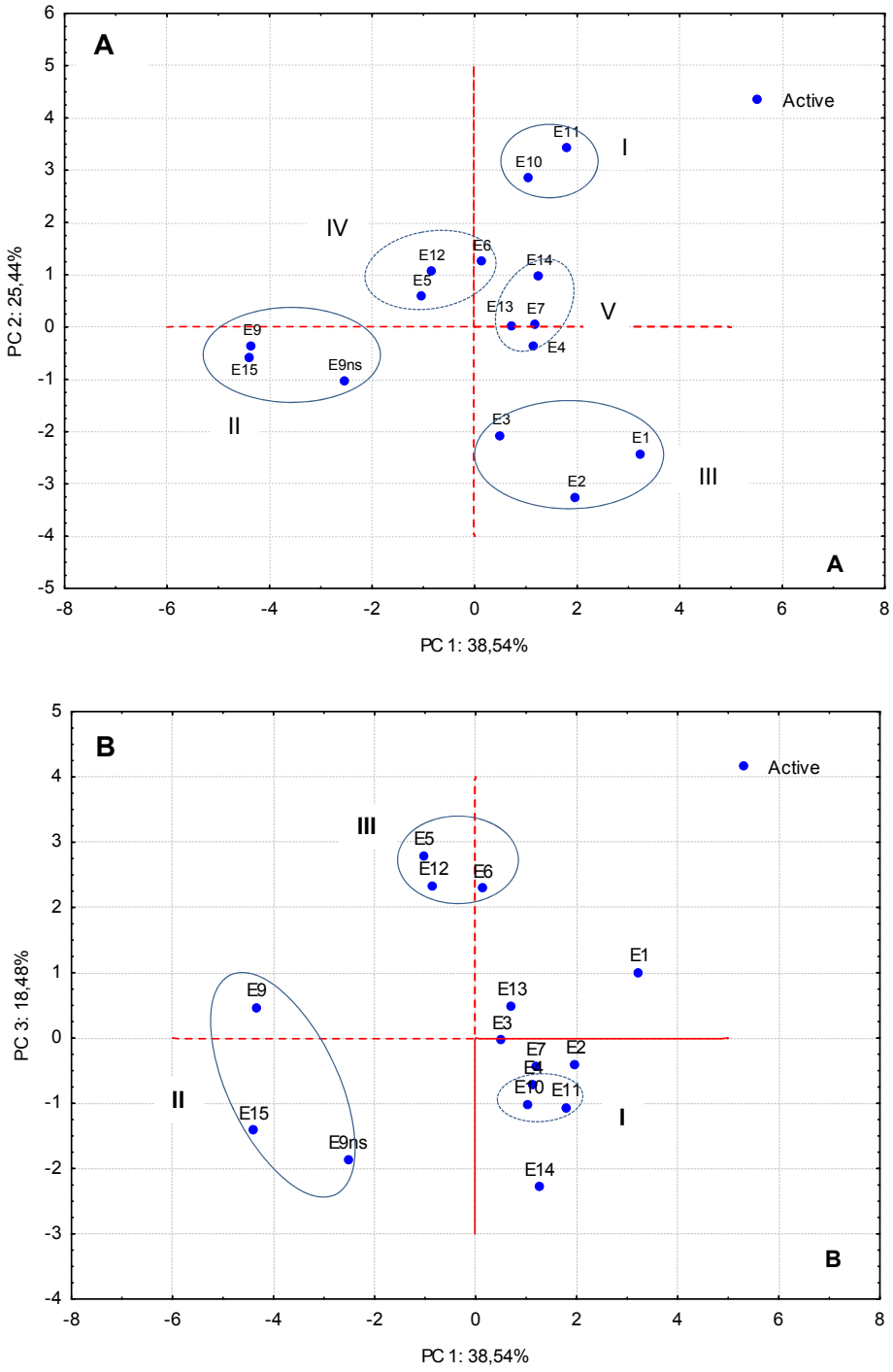

Fig. 2. Projection of the 15 *C. cardunculus* L. ecotypes in the plans defined (A) by principal components PC1 and PC2 and (B) by principal components PC1 and PC3, based on the average results of the 13 technological characteristics, showing a tendency of ecotype aggregation into 3 main groups (I; II; III), with a fourth group that can be divided into two additional groups (panel A: IV and V).

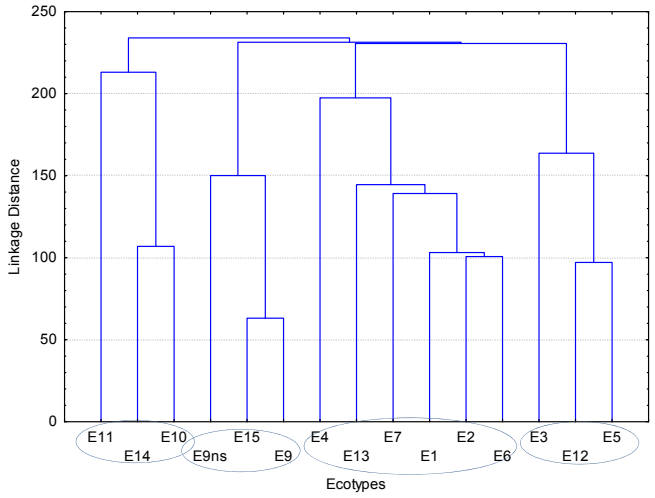

Fig. 3. Dendrogram obtained after Cluster analysis (Euclidean distance, single linkage) using the milk clotting properties evaluated for the flower from the 15 *C. cardunculus* L. ecotypes (dataset of Table 1).

the ecotypes were visibly grouped by the analysis into three clusters (I, II, III) clearly defined, with a fourth cluster of ecotypes located on the centre of the diagram, which can be divided into two clusters (IV and V).

Combining the results of Table 1 with PCA results, cluster I placed in the upper right quadrant of the ecotype distribution contain the ecotypes E10 and E11, which demonstrated higher milk clotting time (R) with lower MCA, poor milk clotting properties (low micellar aggregation rate and low curd firmness), high non-specific PA although showing interesting cheese yield potential. Cluster II, placed on the middle left side of the ecotype distribution, included the ecotypes E9, E9ns and E15 that showed medium R, medium MCA, high micellar aggregation rate/firm curds/good milk clotting properties, medium level of non-specific PA of the enzymatic complex. The cluster III, at the lower right quadrant, assembled the ecotypes E1, E2 and E3, presenting medium R, higher MCA, medium milk clotting properties/low micellar aggregation rate, low level of non-specific PA of the enzymatic complex, and low potential cheesemaking yield. For the central cluster IV, formed by the ecotypes E5, E6 and E12, low MCA with interesting R, good milk clotting properties and high cheesemaking yield were demonstrated. The cluster V, encompassing ecotypes E4, E7, E13 and E14, was discriminated by medium MCA and R; interesting curd properties and high cheesemaking yield. The projection of ecotypes in the plan defined by PC1 and PC3 (Fig. 2B) keep the definition of clusters II and III while all the other ecotypes are joined in the centre of the graph.

The PCA therefore grouped the 15 ecotypes characterised by the variability that Table 1 showed in clusters of closest technological properties. Another multivariate statistical method, the hierarchical cluster analysis (HCA) clearly evidences 4 main clusters of ecotypes (Fig. 3) and confirms most of the ecotype similarities from PCA. Three of the HCA clusters were similar to the first three PCA clusters and the other one joined the last two PCA clusters.

As concluded by Barracosa et al. (2018a,b) for plant morphological characters and for flower cardosin content and profile, our results suggest some diversity also in terms of the technological properties of the different ecotypes. Clusters IV and V seem to be those that encompass the most balanced ecotypes regarding the different aspects evaluated, while for the first three groups some dominant properties are identified. For example, the most important properties for Group III discrimination was MCA, together with an estimated lower PA. For this group, in addition, our results showed a lower potential cheese yield.

There are only a very few studies considering the prediction of cheese properties based on this type of technological properties of cardoon flower although Amira et al. (2017) have recently reviewed the role of plant milk-clotting properties in cheesemaking. Ordiales et al. (2013) associated the type of ecotype properties of cluster III to higher creaminess, viscosity and overall acceptability of Torta del Casar cheese. The extracts with high proteolytic activity negatively influenced the acidity, bitterness, and creaminess parameters. Consequently, it was concluded that the most appropriate cardoons for making this type of cheese are those with higher milk clotting activities and moderate proteolytic activities, especially on β-casein.

The conjugation of the different properties studied may have some significance in the cheesemaking process and on the development of cheese characteristics along the ripening period. However, it is important to note that cheese properties depend also on factors other than the coagulant proteases (Amira et al., 2017). The milk and cheese microbiota and the cheese environment conditions that they contribute to create and under which the action of the proteases will proceed are examples of factors that contribute to define the cheese properties.

4. Conclusions

Based on a survey of cardoon ecotypes of the Alentejo region, in Portugal, the aim of this work was to study the variability of the cheesemaking properties of cardoon flower using a set of technological properties including the effect on cheesemaking yield estimation and milk coagulation properties in addition to the milk clotting and proteolytic activities evaluation.

In addition to the diversity recently found for cardoon plants and for flower cardosin concentration and enzymatic profile, this work identified also some variability on technological properties of cardoon flower collected in the Alentejo region. The multivariate analysis allowed the integration of all the information provided by the analytical protocol used. It was possible to identify differences between clusters of ecotypes according to the studied technological properties, which may have a significant influence in cheese manufacture and on cheese properties.

To conclude for the real significance of differences on flower technological properties in cheesemaking, subsequent studies on the enzymatic profiles associated with the ecotypes and the performance of cheesemaking tests are therefore required. Both studies are ongoing in the frame of the project ValBioTecCynara to better characterise the ecotypes flower and to evaluate their possible effects on the cheese properties allowing the selection of the best flower pattern for traditional cheeses from Alentejo or other cheesemaking regions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2018.09.010.

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