

Article

Profile of Carotenoids and Tocopherols for the Characterization of Lipophilic Antioxidants in “Ragusano” Cheese

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Abstract: Lipophilic antioxidants such as carotenoids and tocopherols are appreciated components in food because of their potential health benefits. The aim of this study was to describe the composition of these microconstituents in “Ragusano”, a typical Sicilian historical *pasta filata* cheese, and to compare them during two different production seasons. Specifically, the tocopherols’ composition was evaluated by high-performance liquid chromatography coupled to a fluorescence detector (HPLC-FD); whereas the contents of three main carotenoids were determined by high-performance liquid chromatography coupled to a diode array detector with atmospheric pressure chemical ionization and mass spectrometry (HPLC-DAD-APCI-MS). The scope included studying the influence of dietary supplementation on the potential enrichment of “Ragusano” in antioxidants. The main results regarding the composition of lipophilic vitamins of 56 “Ragusano” cheeses, collected in winter and spring, revealed that α -tocopherol was the predominant component amongst tocopherols and carotenoids, while β -carotene prevailed among the carotenoids. The cheeses obtained in spring turned out to contain larger amounts of antioxidants, both tocopherols and carotenoids, while the dietary supplementation with minerals-vitamins led to a barely detectable increase of antioxidants compared to a measured control group.

Keywords: Ragusano; tocopherols; carotenoids; cheese; food analysis; HPLC analysis



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1. Introduction

Many Italian cheeses are traditional specialties, which have strong links with their region of origin. The protected designation of origin (PDO) is a trademark of the legal protection of the denomination that is attributed by the European Union to foods whose peculiar qualitative characteristics essentially or exclusively depend on the territory in which they are produced [1]. The PDO brand signifies the highest standards of origin, production, and quality and displays all the preciousness hidden within but visible outside the cheese. In today’s world, there are only a few foods still produced in this way, which is why the PDO brand of cheese is reserved only for those made exclusively with milk produced in the region of origin, the same where it will then be transformed into cheese and finally seasoned.

“Ragusano” is a semi-hard, brine-salted, *pasta filata* cheese produced exclusively from *Modicana* breed cow’s milk. It is one of the oldest cheeses in Sicily and is classified amongst the historical dairy products in the Sicilian region. This cheese, with a sweet and peculiar flavor, has been the object of a flourishing trade beyond the borders of the Sicilian Kingdom since the XIV century [2]. In 1955, it was endorsed with the recognition of typicality

according to the DPR n. 1269 of 30 October 1955 [3]. In 1996 it was recognized with the protected designation of origin (PDO) with EEC regulation n. 1263 of 1/7/1996 [4]. The name “Ragusano” likely derives from the production area, the Province of Ragusa [2], even if the milk processed into “Ragusano” cheese originates from several municipalities of the Iblean plateau near Ragusa and Syracuse.

“Ragusano” PDO is manufactured following an ancient traditional method [5] reproduced by the current manufacturers. In compliance with this practice, “Ragusano” cheese is made in small factories or farms exclusively with whole raw cow’s milk, using the milk’s natural microflora as a coagulation starter. It has a smooth, thin, compact crust with golden yellow or straw yellow color, tending to brown as it ages; the interior is white, tending to straw yellow, and has a compact structure, with possible cracks increasing with aging. Generally, “Ragusano” is produced from October to May, but it is possible to find it seasoned all year round.

The popularity of this cheese among consumers is linked to the taste, pleasant, sweet, delicate, not very spicy in the first months of curing, but turns to spicy and savory after advanced curing. Today, although this cheese is produced according to the official protocol of “Ragusano” PDO, cheese-makers are often inclined to experiment with new dietary supplementation, to remain economically competitive and improve the cheese quality [6]. Thus, the study of the chemical composition is useful both to characterize this historical cheese and to outline the quality originated from the admixture of the natural microflora and traditional cheese-making technology.

In a recent comprehensive review, it was highlighted that cheese, one of the major fermented dairy products, owns a peculiar antioxidant capacity due to the presence of different classes of compounds such as sulfur-containing amino acids, enzymatic systems, and vitamins. Particularly, vitamins A and E are mentioned as “primary lipid-soluble antioxidants” able to efficiently inhibit polyunsaturated fatty acid peroxidation and raise shelf stability [7]. However, less attention has been paid to the content of lipophilic vitamins of “Ragusano”. Usually, the studies performed to characterize and protect the quality of this cheese were devoted to the volatile flavor compounds, sensory properties [6,8–15], fatty acids composition [16,17] or peptide profiles linked to bitterness taste [18]. These data often refer to the manufacturing process (including salt concentration, time, temperature, and pH) [19–21]. To date, no paper is available in the literature on the determination of lipophilic compounds such as tocopherols and carotenoids in “Ragusano” cheese. Currently, the only available source refers to the yellow color index, originating from fresh plant materials, that could be associated with β -carotene and other related carotenoid compounds [9].

Tocopherols (also known as vitamin E) and carotenoids are fat-soluble microconstituents of numerous dietary sources. Some potential benefits that have been attributed to these compounds in humans include protection against cardiovascular disease, cancer, and age-related eye diseases [22,23]. Vitamin E and carotenoids are involved in sensory and nutritional cheeses’ distinctiveness, and their concentrations and relative compositions are often related to genetic and physiological factors, the animal’s forage diet, metabolism by the animal tissues, original milk composition, and cheese-making processes, which are also dependent on several environmental factors [24–26]. Studies, characterizing different cheeses, examine the quality of dairy products and their consumer’s acceptability, which often depends on lipid autoxidation. In this context, tocopherols and carotenoids act as fat-soluble antioxidants and contribute to the oxidative stability of the product [25]. Particularly, it was shown that among ruminants, only bovine accumulate high concentrations of carotenoids (mainly β -carotene and lutein) [25], while α tocopherol is the most representative among vitamin E group [26].

Additionally, in recent years, the importance of the type and quality of fodder in the diet of cows for lactation has received much emphasis, and several studies have shown that the concentration of vitamins in milk and dairy products vary according to the nature of the animals’ foraging [26].

The objective of this research was to study the “Ragusano” composition and determine the carotenoids (α -carotene, β -carotene, and lutein), and vitamin E (α -tocopherol, δ -tocopherol, and γ -tocopherol) contents, to protect the quality of this Italian cheese. Particularly, this investigation is aimed to track possible changes in the liposoluble antioxidant profile by following the cow-feeding and cheese-production seasons. The second objective of this study was inspired by specific strategies that Italy (among the top ten cheese producers in the world) is pursuing to progressively implement innovative guidelines to enhance dairy quality toward both the consumers’ health and the supply chain’s sustainability. Furthermore, since dairy management can affect the final cheese composition, the second objective was to investigate the seasonal variation of micro-constituents in “Ragusano” using a mineral-vitamin dietary supplementation.

2. Materials and Methods

2.1. Chemicals

Methanol, *n*-hexane, and ethyl acetate were UHPLC/MS-grade from Optima, Fisher Chemical products (Milan, Italy). All other reactants and solvents, tocopherols (α -tocopherol, δ -tocopherol, and γ -tocopherol) and carotenoids (α -carotene, β -carotene, and lutein) were purchased from Sigma-Aldrich (Milan, Italy). Poly-tetra-fluor-ethylene (PTFE) syringe filters (0.45 μ m) were from Gelman Sciences Inc. (Ann Arbor, MI, USA), while 0.2 μ m OlimPeak syringe filters with nylon membranes were purchased from Teknokroma (Monza Brianza, Italy).

2.2. Samples and Cheesemaking

Fifty-six “Ragusano” PDO cheese samples were obtained directly by a producer from the Ragusa area, and the collection regarded two different production seasons (winter and spring) in 2020. Cheese samples were produced using milk from 14 selected *Modicana* breed cows, multiparous in lactation, to evaluate the effect of the production season (winter or spring) on the content of carotenoids and tocopherols in the cheese. Due to problems related to cow lactation between winter and spring, some “Ragusano” cheese samples manufactured in spring were made from the milk of different cows. All the experimental procedures used were performed in compliance with the European Union guidelines on animals in research [27].

In order to avoid any possible data artifacts owing to different breeding and manufacture practices, the study was conducted in a farm, and the results correspond to a semi-extensive breeding system, which is a very widespread cattle breeding practice in Sicily. “Ragusano” cheese was produced with whole raw cow’s milk, and the feeding of the cows consisted mainly of grazing natural pastures in the Hyblaean territory. The cows grazed freely on a natural pasture of approx. 20 ha and had free access to drinking water. In winter (between January and February), the cows were additionally fed hay and silage. The spring pasture (between March and April) presented a remarkable richness of forage essences at the juvenile’s stage. In both seasons, the cows received a food supplementation in the stable, which consisted of local cereals and vegetables (corn, soybean meal, wheat flour, carob germ, beet pulp, and carob pulp). Seven *Modicana* cows were selected for each production season, and their diet was supplemented with a premix of minerals (Mn, Fe, Zn, Cu, and Se) and vitamins (A, D3, and E). The cheese samples obtained from *Modicana* cow milk without mineral-vitamin supplementation were conventionally designed as control (C). The cheese samples from *Modicana* cows that received the same basal diet, and mineral-vitamin supplementation were conventionally considered test (T) samples. For each *Modicana* cow, two cheese samples were manufactured and collected at two different ripening stages: at the beginning of the investigation, when no supplementations were used (C0 and T0), and at the end of the experiment, 30 days after the use of mineral-vitamin supplementation (C1 and T1). From each cheese sample, aliquots of 30 g were cut from the inner part, vacuum-wrapped, and stored at -40 °C until analysis to promote the long-term stability of the lipophilic vitamins.

The cheese samples were manufactured following the indications established by the production discipline [4]. The milk from one or more milking cycles was coagulated by adding kid or lamb rennet paste, along with water and salt, favoring the natural development of the microflora. The curd was then reduced into small pieces using the “iaruozzu”, a wooden rod that ends with a disc and subjected to pressing to facilitate sponging. The curd was then covered to avoid sudden drops in temperature (about 80 °C) and left to rest for 85 min. The drying took place by depositing the curd in the “vasceddi”, a small basket from which the whey was dripped. Subsequently, a second cooking was carried out, again at 80 °C, and the curd was left to rest for twenty hours, the time necessary to obtain the right degree of acidity. After fermentation, the thick paste was cut into slices and placed in the sieve, where it was treated with hot water. The product was then collected in wooden containers called “mastredde”, which give “Ragusano” PDO its characteristic shape. After the brine-salting, the aging (30 g) of “Ragusano” PDO took place in the “maiazzè”, which are underground, humid, and ventilated rooms, where the temperature was between 14 and 16 °C.

2.3. Sample Pre-Treatment

Before analysis, the cheese samples were defrosted at 4 °C, cut into cubes, stored in beakers, and freeze-dried for 72 h. Containers were protected from light with aluminum foil. Later, the samples were finely crushed, homogenized, and stored at −20 °C in dark plastic tubes until analyses.

2.4. Tocopherol Extraction and Determination by High-Performance Liquid Chromatography Coupled to a Fluorescence Detector (HPLC-FD)

The concentrations of α -, δ -, and γ -tocopherol (i.e., vitamin E) in cheese samples were determined by adapting an analytical method that was recently developed for milk sample analyses [28]. The outcomes were compared with those obtained according to the Havemose et al. [29] method, and the results showed that our procedure is reliable, quick, and has good recoveries. Briefly, 2 g of lyophilized cheese were poured into a dark screw-cup Pyrex glass test tube, added with *n*-hexane (2 mL), and homogenized (10 s at room temperature). Then 2 mL of potassium hydroxide methanolic solution (2M) were added, the oxygen removed with nitrogen flow, the vessel capped, and the resulting mixture mixed (10 s at room temperature). After that, the final mixture was sonicated at 20 °C for 10 min and subsequently centrifuged (10 min at 4000 rpm, 4 °C). The supernatant was isolated, filtered through a 0.2 μ m OlimPeak syringe filter with nylon membrane (Teknokroma, Monza Brianza, Italy), transferred into an amber vial, and analyzed by HPLC/FD.

Chromatographic analysis was performed using a Shimadzu (Milan, Italy) HPLC system LC-20AD equipped with a fluorescence detector RF-20A, a CBM-20A controller, a CTO-20A column oven, one LC-20AD pump, and a DGU-20A3 degasser. The analyses were performed using a *LiChrosorb*[®] *Si 60* (5 μ m) column (4.6 mm I.D. \times 250 mm), protected by a guard column with the same stationary phase. Analyses were run at 40 °C in isocratic conditions using a mobile phase composed of *n*-hexane/ethyl acetate (90:10 *v/v*). The injection volume was 20 μ L, and the flow rate was 0.8 mL/min. Fluorescence excitation and emission wavelengths were 295 nm and 330 nm respectively, and α -, δ -, and γ -tocopherol were identified using commercial standards.

The quantitative analysis was carried out with the standard external method using suitable calibration curves and any quantification estimated as the mean value of three repeated measurements and referred to the cheese samples before the lyophilization process. The results are expressed as mean \pm standard deviation.

2.5. Carotenoid Extraction and Analysis by High-Performance Liquid Chromatography Coupled to a Diode Array Detector with Atmospheric Pressure Chemical Ionization and Mass Spectrometry (HPLC-DAD-APCI-MS)

Carotenoids (α -carotene, β -carotene, and lutein) were extracted from cheese lyophilized samples adjusting an extracting method proposed by Stout et al. [30] for bovine milk sam-

ples, and all treatments were performed under subdued light. In short, in a test tube, 3 g of lyophilized sample, added with ethanol (3 mL) and a saponification solution (3 mL of KOH 25% *w/v* in deionized water), were fluxed with nitrogen, capped, vortexed (1 min) and subjected to ultrasound-assisted treatment (5 min at 20 °C). Then, the mixture was heated at 40 °C for 30 min for the saponification. Following, 6 mL of *n*-hexane/ethyl acetate (70:33, *v/v*) were added, and after another vortex-stirring (1 min) and sonication (5 min) treatment, the sample was centrifuged (10 min at 800 rpm, 25 °C) and the upper layer was collected. The ultrasound-assisted extraction was repeated with a fresh *n*-hexane/ethyl acetate mixture for three cycles; the upper organic phases were pooled and dried under nitrogen. The residue was dissolved in 1 mL of methyl-terz-butyl-ether (MTBE)/methanol (1:1, *v/v*), filtered through 0.45 µm PTFE, and analyzed by HPLC following a method we used for other carotenoid determinations [31,32].

The analyses were carried out using an HPLC system (Shimadzu, Milan, Italy) equipped with an SPD-M20A photo-diode array detector, two LC-20AD pumps, a CBM-20A controller, a DGU-20A3 degasser, and a SIL-20AC auto-sampler. The data were processed with the software LabSolutions ver. 5.10.153 (Shimadzu, Milan, Italy). For MS analyses, the system was coupled to a mass spectrometer detector (LCMS-2020, Shimadzu, Milan, Italy), equipped with an APCI interface, both in positive and negative ionization mode. Carotenoids were separated on a C30 YMC column (250 × 4.6 mm; 5 µm), and the injection volume was 20 µL. The mobile phase was a binary gradient of methanol/methyl-terz-butyl ether/water (MeOH/MTBE/H₂O) (90:8:2; *v/v/v*) (A), and MeOH/MTBE/H₂O (8:90:2; *v/v/v*) (B): 0.01–20.00 min, 0–30% B; 20.01–35.00 min, 30–80% B; 35.01–65.00 min, 100% B; 65.01–75.00, 100% B. Then, the gradient was re-equilibrated to the initial condition in another 5 min before the next analyses. The flow rate was 1 mL/min, and the analyses were performed at 20 °C. Pigments (α -carotene, β -carotene, and lutein) in cheese samples were identified, comparing elution order, UV/vis, and APCI-MS spectra with those of available standards. The UV/vis spectra were acquired in the range of 250–600 nm, while the chromatograms were extracted at 448 nm for β -carotene, 444 for lutein, and 325 for α -carotene (sampling frequency: 1.5625 Hz; time constant: 0.64 s). The MS/APCI parameters were set as follows: Acquisition Mode, Scan; Interface Temperature, 350 °C; Interface Voltage: 4.5 kV; Heat Block, 300 °C; CDL Voltage: 0 V; CDL temperature, 300 °C; *m/z* range, 300–1200; Nebulizing gas flow (N₂): 4 mL/min; Event Time: 1 s; Detector Voltage: 0.8 kV; Q-array: 0.0 V; RF: 90 V; Sampling: 2 Hz.

The identified compounds were also confirmed by comparing the spectral data of authentic standards, while quantification was performed with the external standard method. The data on the pigment levels referred to the cheese samples before the lyophilization process and were obtained as average values of triplicate analyses, and the results are expressed as mean \pm standard deviation. The coefficient of variation (CV%) of the three analyses was always lower than 5%.

2.6. Statistical Analysis

Results were rationalized using multivariate statistical approaches to graphically track the variation of the six analyzed metabolites. Specifically, the PCA (principal component analysis) performs a dimensional reduction with respect to the number of quantitative variables showing, in a 2D graph, the samples as spots (scores) spread in the plot according to the variance of their detected quantitative values (known as loadings). We used Matlab (1994–2021 The MathWorks, Inc., Milan, Italy) and R software for statistical analysis, and the graphs were obtained using these software programs. PCA is the simplest statistical evaluation of the variances working without any given information about the origin of the samples (unsupervised mode); this ensures no artifacts in the performed evaluations.

We proposed the projection of the variance of several variables (in our case, six) along two common vectors (principal components) on a plane matching the shared variabilities [33]. In order to represent a comprehensive view of the general variance concerning the six measured variables, we used a multivariate statistical approach (MVA) to graph-

ically track the variation of variables (loadings) over several observables (scores). The modern unsupervised approach called PCA (principal component analysis) [34] performs a dimensional reduction to follow, at a glance, more quantitative variables (in this case, six loadings). 2D plots (called biplots) show just the first two principal components, displaying a pretty clear trend of the variables, to interpret the difference between 56 cheese samples produced in two different seasons (Figure 1 explaining the 92.7% of the total variance) and 28 cheese samples in the same season obtained at the beginning of the study and after two different diet regimes (Figure 2 explains the 87.9% of the total variance for winter variables and Figure A1 explains the 90.4% of the total variance). Scores are spread in the 2D space according to the variance of their detected quantitative values along with the principal components. All the calculations and graphical representations were created in Matlab (1994–2021 The MathWorks, Inc., Milan, Italy) and R software for statistical analysis. PCA is the simplest statistical evaluation of the variances working without any information about the origin of the samples (unsupervised mode); this ensures no artifacts in the visualization and evaluation.

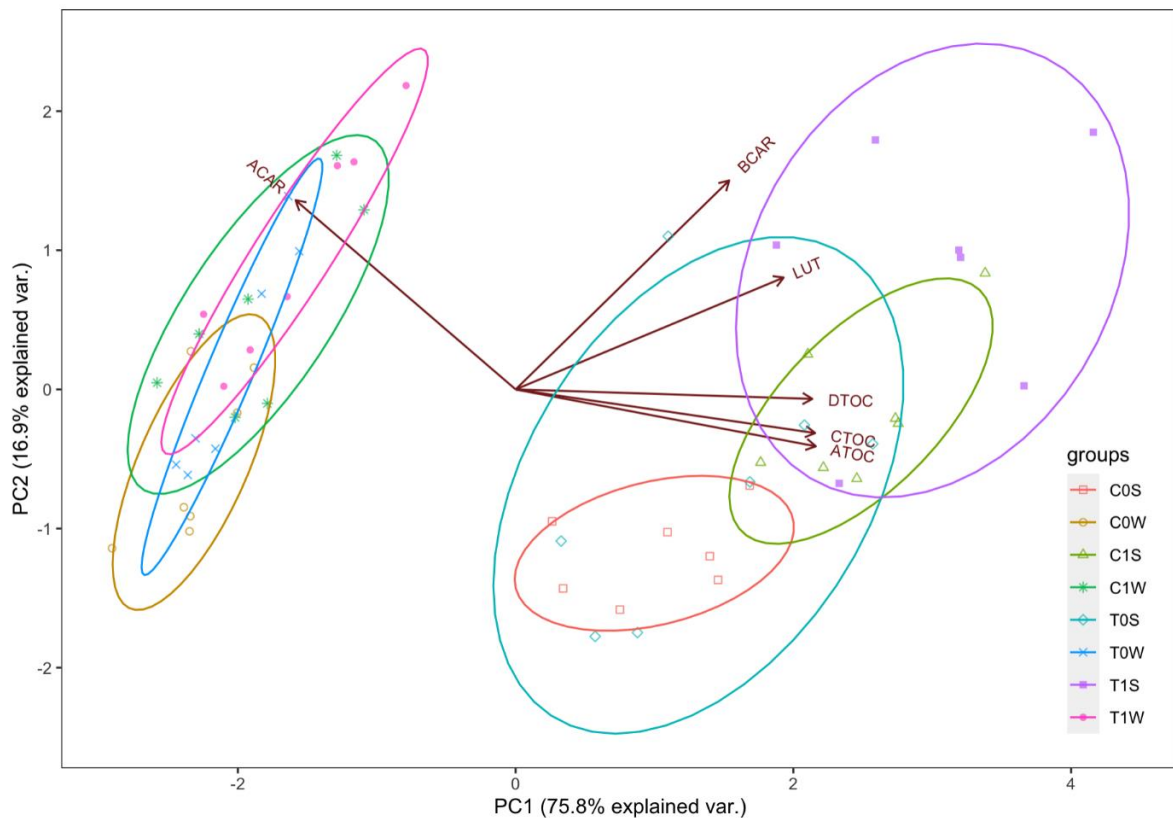


Figure 1. Biplot 2D—graph for the principal component analysis (PCA) of all 56 samples of “Ragusano” cheese; the first two principal components represented as PC1 and PC2 explain 92.7% of the total variance. Vectors represent the general variance of the variables (loadings) with respect to the samples represented by spots (scores) distributed in the graph. Even in this unsupervised scattering mode, it is evident the statistical trend of the six antioxidant species, moreover, ellipses clusters samples coming from control (C) or enriched (T) diet; before (0) or after (1) grazing pasture; produced in winter (W) or spring (S).

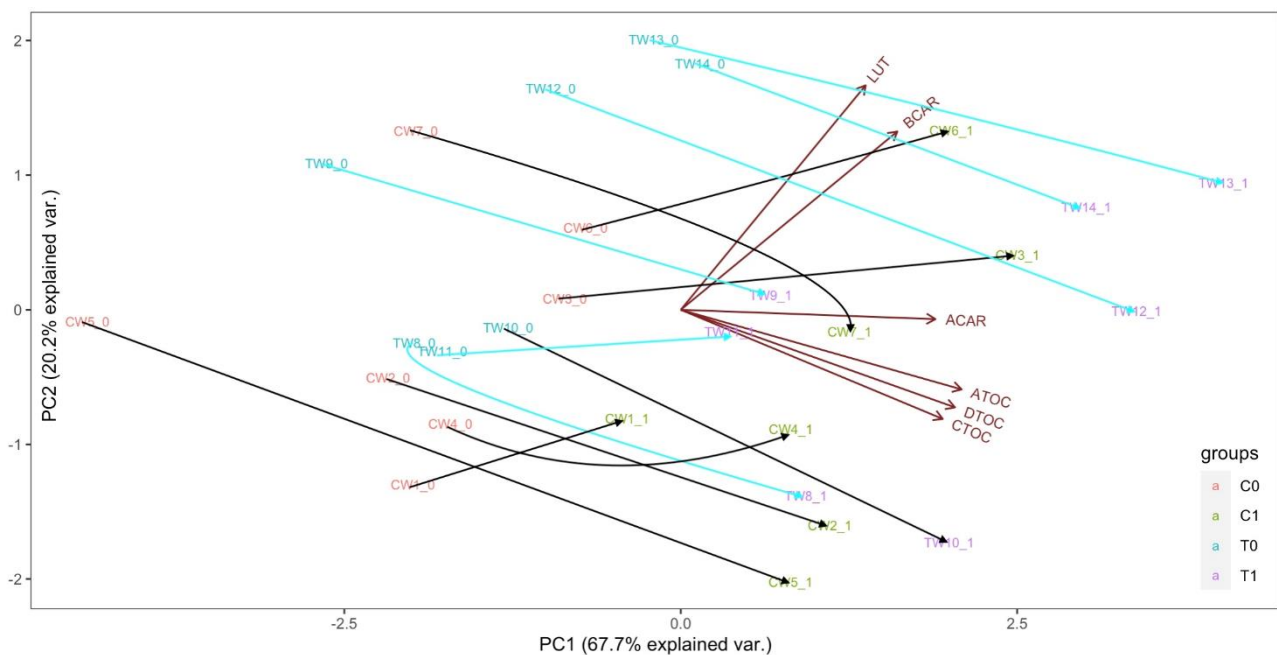


Figure 2. Biplot of 2D–graph of PCA for the 28 winter produced samples. The six variables are projected along with the first two main components, PC1 and PC2, which, in this case, account for 87.9% of the total variability. We chose to highlight with lines the shift of cheese sample coming from the same cow, before (₀ spots) and after (₁ spots) the grazing feeding.

3. Results

This study has produced the first fingerprint of tocopherols (TOCs: α -, δ -, and γ -tocopherol) and carotenoids (CARs: α -carotene, β -carotene, and lutein) in “Ragusano” cheese, a traditional Italian cheese whose identity must be preserved. Using the protocols described before, these microconstituents were determined, and Tables 1 and 2, along with Figures 1 and 2, show the results observed on the cheese samples manufactured by milk produced individually by each *Modicana* cow at the start of the trial (T = 0) and at the last day of the study (T = 1). The extended results are reported in Appendix A as Figure A1 and Tables A1 and A2. The data grouped according to the different production seasons (winter and spring) and the mineral-vitamins supplementation highlight that the production season significantly affected all the determined analytical parameters.

Remarkably, as Table 1 shows, α -tocopherol (α -TOC) was the predominant component amongst TOCs and CARs. We found that α -TOC accounted on average for 62.24% and 57.73% amongst the total TOCs in the winter and spring cheese samples, respectively (Table A1). The average total TOC content increased significantly from winter (37.73 ± 6.73 mg/kg) to spring (103.92 ± 17.23 mg/kg), even if the indices ratio of tocopherols was maintained across the seasons. The winter content of α -TOC (total range: 17.30–28.46 mg/kg) was nearly tripled in “Ragusano” samples obtained in spring (total range: 45.60–70.81 mg/kg). Similarly, δ - and γ -tocopherol (δ -TOC and γ -TOC) concentrations were almost doubled, and sometimes tripled, in spring-cheeses with respect to the winter analogs.

The “Ragusano” CARs’ composition showed that the analyzed compounds were subjected to variation from one season to another. Generally, their contents increased in the cheese samples produced in spring even if the increment was not so high as for tocopherol (especially α -TOC). For instance, Table 1 shows a general increase in the content of total carotenoids, whose average values passed from 11.52 mg/kg of the winter to 15.38 mg/kg of the spring. Mainly, the average relative ratio amongst all the carotenoids analyzed remains almost unaffected as the seasons change.

Table 1. Occurrence of tocopherols (α -, δ -, and γ -tocopherol) and carotenoids (α -carotene, β -carotene, and lutein) in “Ragusano” cheese samples produced in the winter. Concentrations (mg/kg fresh weight) of single compounds are expressed as mean \pm s.d. ($n = 3$).

Sample ¹	α -TOC ²	γ -TOC ²	δ -TOC ²	Total TOCs ²	LUT ²	α -CAR ²	β -CAR ²	Total CARs ²
CW1_0	23.10 \pm 1.72	9.61 \pm 1.04	3.03 \pm 0.30	35.74	2.11 \pm 0.28	0.50 \pm 0.04	5.80 \pm 0.62	8.41
CW2_0	20.72 \pm 1.68	9.02 \pm 0.80	3.04 \pm 0.27	32.78	2.82 \pm 0.38	0.56 \pm 0.05	5.05 \pm 0.46	8.43
CW3_0	22.06 \pm 1.95	9.64 \pm 0.95	4.01 \pm 0.46	35.71	3.50 \pm 0.35	0.64 \pm 0.05	6.42 \pm 0.55	10.56
CW4_0	23.02 \pm 1.67	9.01 \pm 0.86	3.70 \pm 0.34	35.73	2.80 \pm 0.33	0.57 \pm 0.05	4.82 \pm 0.41	8.19
CW5_0	17.03 \pm 1.27	6.22 \pm 0.64	1.23 \pm 0.16	24.48	2.11 \pm 0.30	0.51 \pm 0.05	4.74 \pm 0.45	7.36
CW6_0	22.71 \pm 1.74	8.73 \pm 0.86	4.20 \pm 0.41	35.64	3.70 \pm 0.30	0.66 \pm 0.05	7.30 \pm 0.69	11.66
CW7_0	18.44 \pm 1.69	7.11 \pm 0.73	2.80 \pm 0.21	28.35	3.72 \pm 0.34	0.75 \pm 0.06	6.62 \pm 0.63	11.09
Range C ₀	17.03–23.10	6.22–9.64	1.23–4.20	24.48–35.74	2.11–3.72	0.50–0.75	4.74–7.30	7.36–11.66
TW8_0	21.35 \pm 1.55	8.60 \pm 1.02	3.00 \pm 0.27	32.95	2.94 \pm 0.30	0.60 \pm 0.06	0.48 \pm 0.63	9.02
TW9_0	19.84 \pm 1.34	7.03 \pm 0.91	2.30 \pm 0.18	29.17	3.60 \pm 0.34	0.53 \pm 0.04	6.03 \pm 0.65	10.16
TW10_0	22.31 \pm 1.56	8.40 \pm 0.97	4.12 \pm 0.44	34.83	3.30 \pm 0.33	0.69 \pm 0.06	5.36 \pm 0.50	9.35
TW11_0	21.80 \pm 1.70	7.62 \pm 0.55	4.36 \pm 0.48	33.78	3.00 \pm 0.29	0.67 \pm 0.06	5.04 \pm 0.50	8.71
TW12_0	20.16 \pm 1.77	8.45 \pm 0.73	3.00 \pm 0.32	31.61	4.02 \pm 0.42	0.70 \pm 0.06	8.59 \pm 0.78	13.31
TW13_0	21.68 \pm 1.43	8.73 \pm 0.81	3.45 \pm 0.29	33.86	4.51 \pm 0.42	0.72 \pm 0.06	9.20 \pm 0.81	14.43
TW14_0	22.14 \pm 1.72	8.55 \pm 0.88	3.80 \pm 0.29	34.49	4.06 \pm 0.36	0.81 \pm 0.08	10.44 \pm 0.99	15.31
Range T ₀	19.84–22.31	7.03–8.73	2.30–4.36	29.17–34.83	2.94–4.51	0.53–0.81	5.04–10.44	8.71–15.31
CW1_1	23.19 \pm 1.85	9.96 \pm 1.06	6.33 \pm 0.50	39.48	2.50 \pm 0.31	0.57 \pm 0.06	8.51 \pm 0.71	11.58
CW2_1	26.61 \pm 1.98	10.40 \pm 0.87	8.46 \pm 0.55	45.47	3.20 \pm 0.37	0.80 \pm 0.07	5.40 \pm 0.58	9.40
CW3_1	27.10 \pm 2.30	11.25 \pm 1.22	7.80 \pm 0.58	46.15	4.13 \pm 0.39	0.79 \pm 0.07	10.58 \pm 0.97	15.50
CW4_1	25.20 \pm 2.20	10.04 \pm 0.95	6.00 \pm 0.62	41.24	3.17 \pm 0.37	0.94 \pm 0.08	6.42 \pm 0.71	10.53
CW5_1	24.05 \pm 1.89	10.60 \pm 1.01	7.60 \pm 0.68	42.25	2.55 \pm 0.34	0.99 \pm 0.09	5.21 \pm 0.55	8.75
CW6_1	26.51 \pm 2.31	8.97 \pm 0.84	7.06 \pm 0.60	42.54	4.39 \pm 0.41	0.89 \pm 0.09	10.77 \pm 0.98	16.05
CW7_1	26.03 \pm 2.39	10.35 \pm 0.90	5.66 \pm 0.47	42.04	3.83 \pm 0.32	0.90 \pm 0.09	7.15 \pm 0.63	11.88
Range C ₁	23.19–27.10	8.97–11.25	5.66–8.46	39.48–46.15	2.50–4.39	0.57–0.99	5.21–10.77	8.75–16.05
TW8_1	25.92 \pm 2.61	10.78 \pm 1.10	6.88 \pm 0.52	43.58	3.05 \pm 0.30	0.83 \pm 0.07	6.15 \pm 0.66	10.03
TW9_1	25.33 \pm 2.23	9.02 \pm 0.85	6.75 \pm 0.49	41.10	3.36 \pm 0.29	0.74 \pm 0.07	9.13 \pm 0.80	13.23
TW10_1	27.88 \pm 1.99	11.54 \pm 0.89	8.30 \pm 0.68	47.72	3.05 \pm 0.32	0.88 \pm 0.08	6.88 \pm 0.59	10.81
TW11_1	24.02 \pm 2.27	9.39 \pm 0.76	4.98 \pm 0.42	38.39	3.44 \pm 0.31	0.92 \pm 0.08	6.79 \pm 0.63	11.15
TW12_1	28.46 \pm 2.51	11.88 \pm 1.17	8.10 \pm 0.76	48.44	4.10 \pm 0.35	0.97 \pm 0.10	10.30 \pm 1.06	15.37
TW13_1	28.40 \pm 2.54	12.01 \pm 1.23	8.70 \pm 0.70	49.11	4.70 \pm 0.52	0.94 \pm 0.08	12.28 \pm 1.03	17.92
TW14_1	27.83 \pm 2.32	11.20 \pm 0.99	7.37 \pm 0.63	46.40	4.60 \pm 0.46	0.92 \pm 0.08	10.27 \pm 1.00	15.79
Range T ₁	24.02–28.46	9.02–12.01	4.98–8.70	38.39–49.11	3.05–4.70	0.74–0.97	6.15–12.28	10.03–17.92
Total range	17.03–28.46	6.22–12.01	1.23–8.70	24.48–49.11	2.11–4.70	0.50–0.99	4.74–12.28	7.36–17.92
Total mean value \pm S.D.	23.40 \pm 3.13	9.29 \pm 1.54	5.07 \pm 2.24	37.73 \pm 6.73	3.42 \pm 0.76	0.74 \pm 0.16	7.36 \pm 2.26	11.52 \pm 2.98

¹ Sample name is four characters: the first defines whether the cow follows a normal diet (C) or an enriched diet (T), the second refers to the production season (W or S), the third figure identifies from time to time the same producing animal, and the fourth figure indicates whether the cheese is produced before (0) or after (1) the studied pasture. ² TOC, tocopherol; CAR, carotene; LUT, lutein. Values are the mean value \pm standard deviation of triplicate analyses.

According to Table A1, lutein (LUT) and β -carotene (β -CAR) were the predominant components among the carotenoids tested and accounted for 30.01% and 63.34%, respectively, in the cheese samples produced in winter. A similar constitution of LUT and β -CAR was observed for the “Ragusano” samples produced in the spring (35.12% and 63.03%, respectively; see Table A2) which, showed the highest LUT and β -CAR amounts, especially at the end of the spring period. This compositional change was more consistent for LUT whose content passed from an average winter content of 3.42 mg/kg (total range: 2.11–4.70 mg/kg) to 5.33 mg/kg (total range: 3.72–7.86 mg/kg) in the spring. Peculiarly, the “Ragusano” compositional variability in α -carotene (α -CAR) from winter (total range: 0.50–0.99 mg/kg) to spring (total range: 0.12–0.48 mg/kg) showed a decrease in spring samples.

Table 2. Occurrence of tocopherols (α -, δ -, and γ -tocopherol) and carotenoids (α -carotene, β -carotene, and lutein) in “Ragusano” cheese samples produced in spring. Concentrations (mg /kg fresh weight) of single compounds are expressed as mean \pm s.d. ($n = 3$).

Sample ¹	α -TOC ²	γ -TOC ²	δ -TOC ²	Total TOCs ²	LUT ²	α -CAR ²	β -CAR ²	Total CARs ²
CS15_0	59.20 \pm 5.01	24.53 \pm 2.10	15.54 \pm 1.23	99.27	4.62 \pm 0.45	0.12 \pm 0.01	7.81 \pm 0.67	12.55
CS16_0	48.80 \pm 3.48	18.60 \pm 1.56	11.35 \pm 0.98	78.75	4.01 \pm 0.44	0.16 \pm 0.01	7.00 \pm 0.59	11.17
CS17_0	56.78 \pm 6.30	20.42 \pm 1.88	13.72 \pm 1.08	90.92	4.20 \pm 0.43	0.17 \pm 0.01	6.55 \pm 0.70	10.92
CS18_0	57.23 \pm 6.15	22.60 \pm 1.76	16.40 \pm 1.42	96.23	4.03 \pm 0.32	0.25 \pm 0.02	8.42 \pm 0.71	12.70
CS19_0	58.24 \pm 7.16	27.65 \pm 2.05	17.01 \pm 1.47	102.90	4.10 \pm 0.34	0.24 \pm 0.03	8.10 \pm 0.78	12.44
CS20_0	45.60 \pm 3.32	17.76 \pm 1.37	10.10 \pm 0.99	73.46	4.84 \pm 0.51	0.26 \pm 0.03	6.93 \pm 0.65	11.92
CS21_0	58.76 \pm 5.41	26.90 \pm 1.94	20.57 \pm 1.13	106.23	4.40 \pm 0.40	0.32 \pm 0.04	9.11 \pm 0.77	13.83
Range C ₀	45.60–59.20	17.76–27.65	10.10–20.57	73.46–106.23	4.01–4.84	0.12–0.32	6.55–9.11	10.92–13.83
TS22_0	47.93 \pm 4.19	16.30 \pm 1.37	9.72 \pm 0.86	73.95	4.72 \pm 0.42	0.18 \pm 0.02	7.23 \pm 0.61	12.13
TS23_0	63.30 \pm 7.08	25.68 \pm 1.98	12.98 \pm 0.99	101.96	3.80 \pm 0.30	0.24 \pm 0.02	6.22 \pm 0.69	10.26
TS24_0	52.43 \pm 5.08	23.12 \pm 1.72	14.43 \pm 1.11	89.98	3.72 \pm 0.32	0.20 \pm 0.02	6.01 \pm 0.65	9.93
TS25_0	58.30 \pm 5.10	28.40 \pm 2.08	16.80 \pm 1.27	103.50	4.65 \pm 0.43	0.28 \pm 0.03	9.34 \pm 0.84	14.27
TS26_0	60.21 \pm 6.12	27.84 \pm 1.99	18.76 \pm 1.36	106.81	5.26 \pm 0.41	0.30 \pm 0.05	10.22 \pm 0.95	15.78
TS27_0	61.06 \pm 6.51	29.61 \pm 1.69	23.20 \pm 1.42	113.87	6.11 \pm 0.50	0.29 \pm 0.03	9.08 \pm 0.99	15.48
TS28_0	49.68 \pm 4.08	16.67 \pm 1.25	7.49 \pm 0.52	73.84	6.33 \pm 0.49	0.39 \pm 0.03	12.44 \pm 1.01	19.16
Range T ₀	47.93–63.30	16.30–29.61	7.49–23.20	73.84–113.87	3.72–6.33	0.18–0.39	6.01–12.44	9.93–19.16
CS15_1	67.28 \pm 6.81	30.30 \pm 2.31	24.15 \pm 1.40	121.73	5.50 \pm 0.53	0.33 \pm 0.04	10.39 \pm 1.12	16.22
CS16_1	55.37 \pm 5.25	23.12 \pm 1.56	19.27 \pm 1.34	97.76	5.12 \pm 0.47	0.21 \pm 0.02	9.66 \pm 1.01	14.99
CS17_1	62.01 \pm 6.02	28.88 \pm 1.74	21.22 \pm 1.18	112.11	5.00 \pm 0.42	0.27 \pm 0.02	9.72 \pm 0.99	14.99
CS18_1	67.80 \pm 5.98	32.16 \pm 2.14	24.30 \pm 1.33	124.26	6.27 \pm 0.48	0.38 \pm 0.04	13.45 \pm 1.16	20.10
CS19_1	65.83 \pm 5.94	30.00 \pm 2.11	21.20 \pm 1.25	117.03	5.62 \pm 0.55	0.27 \pm 0.02	8.96 \pm 0.88	14.85
CS20_1	53.02 \pm 4.53	22.23 \pm 1.68	17.14 \pm 1.36	92.39	7.03 \pm 0.71	0.26 \pm 0.02	10.12 \pm 0.96	17.41
CS21_1	69.04 \pm 5.52	32.30 \pm 2.08	26.19 \pm 1.58	127.53	5.04 \pm 0.39	0.40 \pm 0.04	10.30 \pm 0.94	15.74
Range C ₁	53.02–69.04	22.23–32.30	17.14–26.19	92.39–127.53	5.00–7.03	0.21–0.40	8.96–13.45	14.85–20.10
TS22_1	52.27 \pm 4.28	21.11 \pm 1.72	17.20 \pm 1.39	90.58	5.94 \pm 0.51	0.40 \pm 0.04	13.03 \pm 1.16	19.37
TS23_1	70.81 \pm 5.94	33.85 \pm 1.98	25.83 \pm 1.50	130.49	7.75 \pm 0.68	0.32 \pm 0.03	9.42 \pm 1.02	17.49
TS24_1	65.44 \pm 5.11	29.25 \pm 2.03	23.26 \pm 1.44	117.95	5.00 \pm 0.44	0.30 \pm 0.02	9.17 \pm 0.95	14.47
TS25_1	64.03 \pm 4.86	31.20 \pm 2.01	23.80 \pm 1.52	119.03	6.33 \pm 0.49	0.40 \pm 0.04	13.44 \pm 1.15	20.17
TS26_1	68.43 \pm 6.04	30.43 \pm 1.56	24.61 \pm 1.48	123.47	5.90 \pm 0.55	0.44 \pm 0.05	13.87 \pm 0.92	20.21
TS27_1	69.29 \pm 5.99	34.06 \pm 1.85	26.09 \pm 1.67	129.38	7.86 \pm 0.66	0.48 \pm 0.05	15.11 \pm 1.18	23.45
TS28_1	54.90 \pm 4.35	23.78 \pm 1.41	18.10 \pm 1.17	96.78	7.03 \pm 0.65	0.45 \pm 0.04	14.73 \pm 1.13	22.21
Range T ₁	52.27–70.81	21.11–34.06	17.20–26.09	90.58–130.49	5.00–7.86	0.30–0.48	9.17–15.11	14.47–23.45
Total range	45.60–70.81	16.30–34.06	7.49–26.19	73.46–130.49	3.72–7.86	0.12–0.48	6.01–15.11	9.93–23.45
Total mean value \pm S.D.	58.86 \pm 7.47	25.72 \pm 5.41	18.39 \pm 5.66	102.93 \pm 17.93	5.33 \pm 1.17	0.29 \pm 0.10	9.78 \pm 2.65	15.38 \pm 3.71

¹ Sample name is four characters: the first defines whether the cow follows a normal diet (C) or an enriched diet (T), the second refers to the production season (W or S), the third figure identifies from time to time the same producing animal, and the fourth figure indicates whether the cheese is produced before (0) or after (1) the studied pasture. ² TOC, tocopherol; CAR, carotene; LUT, lutein. Values are the mean value \pm standard deviation of triplicate analyses.

Basically, data processing (Figures 1 and 2) shows that the concentrations of all the analytes were generally lower at the beginning of the study (time T = 0), while the cheeses produced at the end of the trial (T = 1) had a higher average content of tocopherols and carotenoids. As Figure 1 shows, the dietary supplementation with minerals-vitamins did not produce relevant effects on the tocopherols and carotenoids analyzed, although the cheeses were obtained at the end of the experimentation, in spring, are generally richer in antioxidants.

4. Discussion

It is assessed that the “Ragusano” cheese composition is time-dependent, especially with concern to volatile compounds [6]. However, no data are available about other con-

stituents. It is reasonable to assume that within each cheese variety, the conditions of milk production strongly influence the nutritional composition of the cheese [24]. Particularly, the concentrations of lipophilic antioxidants, such as carotenoids (CARs) and tocopherols (TOCs), which might undergo degradation during the cheese production step because of their sensitivity to physical and chemical factors, including air that could vary highly according to the dietary supply [24,25]. Many researchers studied the TOC and CAR concentrations in dairy products in relation to pasture, presence of specific plant species, and relative plant maturity levels [35]; nonetheless, to our knowledge, this is the first study that reports the concentrations of these fat-soluble vitamins in “Ragusano” cheese produced in two different seasons.

It is well known that fresh fodder is rich in LUT, β -CAR, and α -TOC, and their content is positively correlated with the fraction of grass at the juvenile stage and to the leaf/ stem ratio in forage plants [36–38]. Other studies reported that supplementation of β -carotene, increasing plasma concentration of β -carotene and retinol in animals, can exert a positive effect on CARs’ concentrations [25], even though a great variability is observed according to the different CARs’ metabolisms of animal species [39].

Few studies are focused on the relationships between the animals’ feeding system and the liposoluble vitamins’ content in cheese. For instance, a study from Spain reported that the content of α -TOC and α -tocotrienol are affected by the pasture habits of the animals, whereas CARs are not affected [40]. In a similar Sicilian study [41], the semi-extensive program (including grazing) was expectedly related to an enhancement of TOCs and CARs content with respect to intensive (no grazing) breeding. Therefore, it seems evident that the content of carotenoids and vitamin E included in green forages and quality pastures represents a crucial factor affecting antioxidant levels in cheese. These references, together with knowledge of bovine metabolism [37], allowed us to draw some conclusions about the data collected and shown in Tables 1 and 2 and Figures 1 and 2. We considered all 56 analyzed samples to evaluate the remarkable seasonal influence on the antioxidant composition (Figure 1); it looks clear that TOCs are enhanced in spring cheeses with special regard to the α -TOC. From the loadings observation (vectorial variables) in Figure 1, it turns out that (a) the seasonal increase of CARs lutein and β -carotene is less pronounced with respect to the TOCs jumps and (b) the α -carotene follows an opposite correlation being the winter samples those with the highest levels of this metabolite.

The enhancement of antioxidants in spring cheese with respect to the winter product is easily explained by the total grass-based cattle diet providing more antioxidants. On the contrary, in winter, the cattle diet needs to be integrated with hay, which is decreasing the “green” feeding, still featured by the early stage of grass not yet enriched in TOCs. The hay is produced after cropping, collecting, and drying fresh plants in bales, so it is expected to show a remarkable decrease in vitamins [42] and antioxidants [43] due to degradation and photo-oxidation; these statements support our records.

The highest increase of α -TOC levels is explained by the enhancement of photosynthetic tissues in leaves with high α -TOC content [44], which is known to decrease with plant aging. The second most evident enhancement concerns γ -TOC; this information is consistent with the high γ -TOC present in plant flowers and seeds. Therefore, γ -TOC enhancement in spring products shows a pasture shift from the vegetative to reproductive stage [44].

The moderate enhancement of β -carotene and lutein in “Ragusano” obtained in spring is a crucial record which can be traced back to the quality of green pasture. On the other hand, the reduced carotenoid content of cheeses manufactured during the winter season testifies to the limited grazing and consequent drop of CARs in forage, as also evidenced by previous studies [35]. These data should be related to the animal metabolism, as it was already noticed that β -carotene conversion into retinol is also related to the animal genotype [37]; however, the knowledge about *Modicana* cows is still too limited to draw definite comparisons and conclusions.

The α -CAR behavior could seem somewhat unexpected, judging from the general trend; however, we highlight that this metabolite is the least represented among the six analytes (absolute quantities) and is probably less stable under the studied conditions. Thus, the real changes are not dramatic and can reasonably be caused by the easy degradation of α -CAR during the production at higher temperatures in spring.

The intra-season analyses showed a general enhancement of TOCs after any pasture condition; this is expected since cattle were fed a controlled diet, with grazing adding “green” forage. However, the winter samples recorded a remarkable enhancement for TOCs and α -CAR after the three-week forage (Figure 2); whereas the spring samples showed that the enhancement is distributed among all the antioxidants (Figure A1) so that loading vectors are roughly all pointing toward scores (sample spots) collected after the fodder.

For both seasons, half of the samples were selected to study the effect of the enriched diet. By looking at the PCA graphs (Figures 2 and A1), it is possible to observe a slight empowered effect concerning the enhancement expected after forage; however, because of the low number of samples, we cannot draw significant conclusions about the diet. On the other hand, this study can be a starting point to face the crucial challenge of the relationship between dairy products and cattle diet given a specific and peculiar cattle species, such as the *Modicana*.

As stated above, to the best of our knowledge, this is the first study on the quantification of TOCs and CARs in “Ragusano” between two different production seasons. The results for most of the fat-soluble vitamins were almost ten times higher than those determined in similar studies concerning Italian cheeses [45,46]. Nevertheless, it is not possible to make a direct unscaled comparison as these studies concern different cheeses, produced in diverse contexts, with distinct cheese-making procedures, and from several species’ milks (cows’, goats’, and sheep’ milk). Furthermore, the samples were analyzed with different analytical techniques, although sometimes innovative [45].

5. Conclusions

The results show that compositional variability in carotenoids and tocopherols is subjected to variations that reflect the character of the fresh pasture plants.

Cheeses produced during spring, when cows had green pasture, showed the highest levels of the lipophilic antioxidant studied (especially tocopherols), respect to cheese samples produced in the winter season, characterized by poor pasture. The ability of a vitamin-mineral supplementation to exert a positive effect on “Ragusano” antioxidant compounds represents a challenge for future studies. More extensive studies must be performed to confirm the nutritive value of “Ragusano”, while different diet supplements could be considered to improve its antioxidant composition.

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Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Extended details concerning the text.

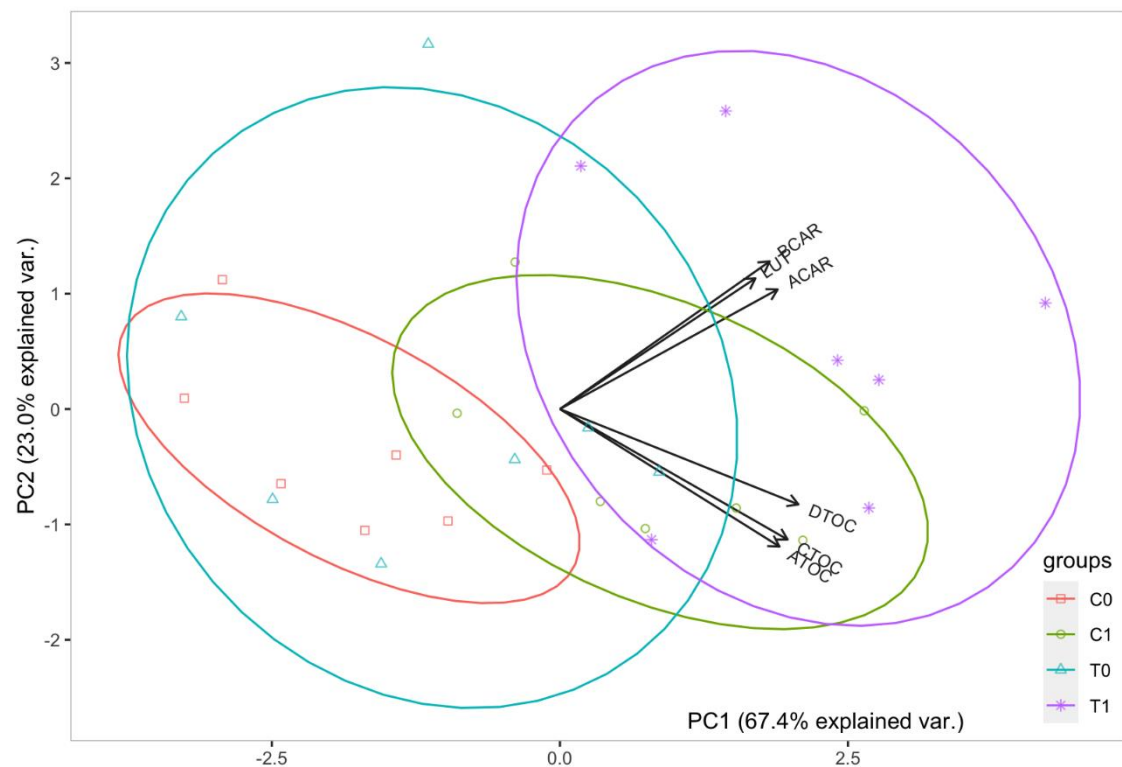


Figure A1. Biplot of 2D–graph of PCA for the 28 spring-produced samples; the shown components explain 90.7% of the total variability. We have chosen to highlight clustering to evidence the slight enrichment of antioxidants after grazing seldom empowered by enriched diet (spots belonging to T1 group). The four groups define control cows (C) and cows with enriched diet (T), after (1) and before (0) the grazing feeding.

Table A1. Percentage of the most representative compounds in “Ragusano” cheeses produced in winter.

Sample	% α -TOC ¹	% LUT ²	% β -CAR ²
CW1_0 ³	64.63	25.09	68.97
CW2_0	63.21	33.45	59.91
CW3_0	61.78	33.14	60.80
CW4_0	64.43	34.19	58.85
CW5_0	69.57	28.67	64.40
CW6_0	63.72	31.73	62.61
CW7_0	65.04	33.54	59.69
Range C0	61.78–69.57	25.09–34.19	58.85–68.97
TW8_0	64.80	32.59	60.75
TW9_0	68.02	35.43	59.35
TW10_0	64.05	35.29	57.33
TW11_0	64.54	34.44	57.86
TW12_0	63.78	30.20	64.54
TW13_0	64.03	31.25	63.76
TW14_0	64.19	26.52	68.19

Table A1. *Cont.*

Sample	% α -TOC ¹	% LUT ²	% β -CAR ²
Range T0	63.78–68.02	26.52–35.43	57.33–68.19
CW1_1	58.74	21.59	73.49
CW2_1	58.52	34.04	57.45
CW3_1	58.72	26.65	68.26
CW4_1	61.11	30.10	60.97
CW5_1	56.92	29.14	59.54
CW6_1	62.32	27.35	67.10
CW7_1	61.92	32.24	60.19
Range C1	56.92–62.32	21.59–34.04	57.45–73.49
TW8_1	59.48	30.41	61.32
TW9_1	61.63	25.40	69.01
TW10_1	58.42	28.21	63.64
TW11_1	62.57	30.85	60.90
TW12_1	58.75	26.68	67.01
TW13_1	57.83	26.23	68.53
TW14_1	59.98	29.13	65.04
Range T1	57.83–62.57	25.40–30.85	60.90–69.01
Total range	56.92–68.02	21.59–35.43	57.33–73.49
Total mean value \pm S.D.	62.24 \pm 3.10	30.01 \pm 3.94	63.34 \pm 4.73

¹ The values are expressed in % with respect to the total tocopherol content; TOC, tocopherol. ² The values are expressed in % with respect to the total carotenoid content; CAR, carotene; LUT, lutein. ³ Sample name is made by four characters: the first figure defines whether the relative cow follows a normal diet (C) or an enriched diet (T), the second one refers to the production season (W or S), the third identifies from time to time the same producing animal, and the fourth figure indicates whether the cheese is produced before (0) or after (1) the studied pasture.

Table A2. Percentage of the most representative compounds in “Ragusano” cheeses produced in spring.

Sample	% α -TOC ¹	% LUT ²	% β -CAR ²
CS15_0 ³	59.64	36.81	62.23
CS16_0	61.97	35.90	62.67
CS17_0	62.45	38.46	59.98
CS18_0	59.47	31.73	66.30
CS19_0	56.60	32.96	65.11
CS20_0	62.07	40.60	58.14
CS21_0	55.31	31.81	65.87
Range C0	55.31–62.45	31.73–40.60	58.14–66.30
TS22_0	64.81	38.91	59.60
TS23_0	62.08	37.04	60.62
TS24_0	58.27	37.46	60.52
TS25_0	56.33	32.59	65.45
TS26_0	56.37	33.33	64.77
TS27_0	53.62	39.47	58.66
TS28_0	67.28	33.04	64.93
Range T0	53.62–67.28	32.59–39.47	58.66–65.45
CS15_1	55.27	33.91	64.06
CS16_1	56.64	34.16	64.44
CS17_1	55.31	33.36	64.84
CS18_1	54.56	31.19	66.92
CS19_1	56.25	37.85	60.34
CS20_1	57.39	40.38	58.13
CS21_1	54.14	32.02	65.44

Table A2. Cont.

Sample	% α -TOC ¹	% LUT ²	% β -CAR ²
Range C ₁	54.14–57.39	31.19–40.38	58.13–66.92
TS22_1	57.71	30.67	67.27
TS23_1	54.26	44.31	53.86
TS24_1	55.48	34.55	63.37
TS25_1	53.79	31.38	66.63
TS26_1	55.42	29.19	68.63
TS27_1	53.51	33.52	64.43
TS28_1	56.73	31.65	66.32
Range T ₁	53.51–57.71	29.19–44.31	53.86–68.63
Total range	53.51–67.28	29.19–44.31	53.86–68.63
Total mean value \pm S.D.	57.73 \pm 3.89	35.12 \pm 3.78	63.03 \pm 3.61

¹ The values are expressed in % with respect to the total tocopherol content; TOC, tocopherol. ² The values are expressed in % with respect to the total carotenoid content; CAR, carotene; LUT, lutein. ³ Sample name is made by four characters: the first figure defines whether the relative cow follows a normal diet (C) or an enriched diet (T), the second one refers to the production season (W or S), the third figure identifies from time to time the same producing animal, and the fourth figure indicates whether the cheese is produced before (0) or after (1) the studied pasture.

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