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Innovative Tools for Nitrogen Fertilization Traceability in Organic Farming Products: A Cauliflower Case Study

Gabriele Campanelli [1], Margherita Amenta [2], Luana Bontempo [3], Fabrizio Leteo [1], Francesco Montemurro [4], Cristiano Platani [1], Nicolina Timpanaro [2], Biagio Torrisi [2] and Simona Fabroni [2,*]

[1] Council for Agricultural Research and Economics (CREA), Research Center for Vegetable and Ornamental Crops, Via Salaria 1, 63030 Monsampolo del Tronto, AP, Italy; gabriele.campanelli@crea.gov.it (G.C.); fabrizio.leteo@crea.gov.it (F.L.); cristiano.platani@crea.gov.it (C.P.)
[2] Council for Agricultural Research and Economics (CREA), Research Center for Olive, Fruit and Citrus Crops, Corso Savoia 190, 95024 Acireale, CT, Italy; margherita.amenta@crea.gov.it (M.A.); nicolina.timpanaro@crea.gov.it (N.T.); biagiofrancesco.torrisi@crea.gov.it (B.T.)
[3] Edmund Mach Foundation, Traceability Unit, Via E. Mach 1, 38098 San Michele All'Adige, TN, Italy; luana.bontempo@fmach.it
[4] Council for Agricultural Research and Economics (CREA), Research Center for Agriculture and Environment, Via Celso Ulpiani, 5, 70125 Bari, BR, Italy; francesco.montemurro@crea.gov.it
* Correspondence: simona.fabroni@crea.gov.it

Abstract: Different research works have been carried out over the years to investigate new and reliable systems to test the authenticity of products obtained using organic cultivation methods. Based on a previously proposed integrated approach for discriminating organic from conventional products through the acquisition of isotopic data and other chemical and biochemical parameters, we herein report the results of an open-field cultivation case study for cauliflower crop. Experiments were carried out on soil, leaves, and corymb samples of cauliflowers grown using six different nitrogen fertilization treatments (organic, conventional, and mixed at different % of mineral fertilizers). The results of this study have shown that a multivariate analysis of isotopic data ($^{13}C/^{12}C$; $^{15}N/^{14}N$, $^{34}S/^{32}S$, $^{2}H/^{1}H$, and $^{18}O/^{16}O$ isotopic ratios) combined with other parameters (fresh weight, total soluble solids, total acidity, cut resistance, CIE L*, a*, b* color indices, head height, head diameter, ascorbic acid content, total polyphenols, and ORAC units) performed using the linear discriminant analysis method gives researchers the possibility to discriminate organic products from conventional ones. Our study highlighted that the different isotopic signatures impressed on the cauliflowers by the different nitrogenous sources combined with the qualitative pattern of the crop, significantly affected by the different treatments, could effectively be jointly used to trace the organic origin of the crop.

Keywords: organic and conventional fertilization; chemical metabolites; isotopic ratios; multivariate analysis; chemometrics

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1. Introduction

According to the FAOSTAT database [1], pesticide use in 2021 was estimated, in terms of use per area of cropland, at 2.26 and 1.75 kg ha^{-1} in the world and in Europe, respectively, while, specifically in Italy, the estimated value was significantly higher, at 5.38 kg ha^{-1}. This is a matter of great concern for consumers who are increasingly sensitive to crop sustainability issues and opens new frameworks for the conversion of cropland to organic agriculture. An organic agriculture system is based on the application of sustainable management practices that aim to increase soil fertility and prevent pests and diseases while rejecting the use of synthetic inputs, such as synthetic fertilizers and plant protection products. The global organic food and beverages market size has been estimated by the Grand View Research Agency [2] at USD 208.19 billion in 2022 and is expected to grow

at a compound annual growth rate of 11.7% from 2023 to 2030. The main reason for the continuously increasing organic market growth is mainly linked to consumer awareness of the beneficial effects of consuming organic foods in their daily diet and their keenness to preserve the health of our planet by promoting a reduction in pesticide use, encouraging the conversion to organic agriculture. The drive towards organic products is also sustained by public institutions and policy guidelines, either at a national, European, or transnational level, following the targets of the European Green Deal [3] and the Sustainable Development Goals introduced by the United Nations [4], which all converge towards the increase in the percentage of organically managed croplands by 2030. Based on the above, the conversion to organic agriculture is currently increasing in all horticultural supply chains, and this demonstrates, in perspective, the great growth potentiality in terms of production volumes of horticultural products that will gain the organic label over the next few years. With regards to the cauliflower supply chain, in 2022 the production value of cauliflower in Italy recorded approximately 315 million EUR on a 14,728 hectares area and a harvested production of 352,064 tons [5]. Regarding organic cauliflower cultivation in Italy, the area was equal to 4302 hectares [6], for an estimated value of approximately 92 million EUR.

Based on what has been stated so far and the high added value of organic products, the fact that organic production may be the object of food fraud carried out by non-virtuous operators and farmers is implied. Indeed, the traceability of organic products is a matter of great interest, and there is a pressing demand from producers, stakeholders, trade associations, federations, and policy makers for new effective tools able to ensure a reliable discrimination between organic and conventional products. The European regulation (EU Reg. 2018/848) that is currently in place [7] only requires documentary evidence of the organic management applied to a product; no official analytical method is recognized or employed as means to support the eradication of food fraud in this specific sector. In an effort to give an answer to these specific needs, several research studies, over almost the last two decades, have focused on the discrimination of organic vs. conventional foods [8–11], including fruit [12–15], vegetable [16–21], meat [22–24], dairy [25–27], and seafood [28] products. The nitrogen fertilization traceability in organic products still remains a matter of great relevance and interest, since synthetic mineral fertilizers are not allowed in organic cultivation, and the need to identify their fraudulent use is pressing. Very recently, an integrated approach has been proposed to evaluate the authenticity of organic produces using isotopic data and other chemical and biological parameters alongside the use of chemometrics [29]. However, at the moment, there is still a lack of knowledge regarding how these new methodologies can be applied in specific and different productive and environmental conditions. As a consequence, with the aim of tracing a methodological path applicable to horticultural products, this research on cauliflower crops was carried out. This research focused on applying a multivariate approach integrated with chemometric tools to discern between cauliflower productions obtained with synthetic fertilizers, typically employed in conventional agriculture and not allowed in organic agriculture, and productions obtained following the organic agriculture method. To achieve this goal, six different treatments were adopted and compared, including conventional, organic, and mixed treatments at different % of mineral fertilizers, also comprising the application of agronomic methods for the management of soil fertility, such as the introduction of agroecological service crops. The variations in the $^{15}N/^{14}N$ isotopic ratio induced by the combination of different fertilization treatments together with the use of agroecological service crops in the organic production system were quantified, thus evaluating how soil management based on an agroecological approach affected the $^{15}N/^{14}N$ isotopic ratio in the final product, in a comparison between the organic management method based on the full substitution of mineral fertilizers and conventional agriculture approaches.

2. Materials and Methods

2.1. Plant Material and Experimental Design

The agronomic trials were performed in two subsequent years (2018–2019 and 2019–2020) at the Council for Agricultural Research and Economics—Research Centre for Vegetable and Ornamental Crops (CREA-OF) of Monsampolo del Tronto (Ascoli Piceno) in Central Italy. The plant material utilized was the late-ripening cauliflower (*Brassica oleracea* L. var. *botrytis*) HF1 Triomphant (Clause seed company), belonging to the white typology. Six fertilization strategies, placed on six nearby fields, were compared. Within each treatment, the fertilization provided 120 units ha^{-1} of nitrogen (N), and the elementary plot was 10.4 m^2, replicated four times. The six treatments, summarized in Table 1, were as follows: (1) "Organic" N fertilization solely with organic animal pellet (3-0-0 of N-P_2O_5-K_2O, respectively) delivered when transplanting the cauliflower on a soil that had not undergone any chemical treatment for several years; (2) "Mix-Organic" treatment on the same soil as treatment 1 and N fertilization with 1/3 organic animal pellets (3-0-0 of N-P_2O_5-K_2O, respectively) at the transplanting stage and 2/3 ammonium nitrate (26-0-0 of N-P_2O_5-K_2O, respectively) at the first mechanical weeding; (3) "Conventional" fertilization with synthetic N using 1/2 YaraMila Blustar (12-12-17 of N-P_2O_5-K_2O, respectively) at the transplanting stage and 1/2 ammonium nitrate (26-0-0 of N-P_2O_5-K_2O, respectively) at the first mechanical weeding, on a soil that had always been managed using conventional techniques; (4) "Mix-Conventional—a" treatment on the same soil as treatment 3 and N fertilization with 1/3 organic animal pellet (3-0-0 of N-P_2O_5-K_2O, respectively) at the transplanting stage and 2/3 ammonium nitrate (26-0-0 of N-P_2O_5-K_2O, respectively) at the first mechanical weeding; (5) "Mix-Conventional—b" treatment on the same soil as treatments 3 and 4 and N fertilization with 2/3 organic animal pellet (3-0-0 of N-P_2O_5-K_2O, respectively) at the transplanting stage and 1/3 ammonium nitrate (26-0-0 of N-P_2O_5-K_2O, respectively) at the first mechanical weeding; and (6) "Organic + Agroecological practices" (Organic + AEP) on a soil that had not undergone any chemical treatment for several years. This last treatment was carried out within the Monsampolo Organic Vegetable Long-Term Field Experiment (MOVE LTE), which is characterized by a four-year vegetable crop rotation. The MOVE-LTE is carried out in a typical thermo-Mediterranean climate, and its soil is a Typic Calcixerepts fine-loamy mixed thermic soil [30], as previously described [31]. The field of the above-mentioned experiment is cultivated under the current legislative framework for promoting the sustainability of food systems (UE 848/2018) and has been managed via an agroecological approach since 2001, also making use of three different cover crops (horseradish, vetch, and a barley/wheat mixture). In the present cauliflower case study, horseradish (*Raphanus sativus* L.) was applied three months before cauliflower transplanting, and N fertilization took place at the transplanting of the cauliflowers only with an organic vegetable amendment (Vegand: 4-1,4-0 of N-P_2O_5-K_2O, respectively).

Table 1. Treatment and fertilization strategies: type of fertilizer, dose, distribution period, and N units.

Treatment	Fertilizer	Dose (kg ha^{-1})	Distribution Period	N (Units ha^{-1})
(1) Organic	Animal pellet (3-0-0) *	4000.00	At transplanting	120
(2) Mix-Organic	Animal pellet (3-0-0)	1333.33	At transplanting	40
	Ammonium nitrate (26-0-0) *	307.69	At the first mechanical weeding	80
(3) Conventional	Multielement synthetic "YaraMila Blustar" (12-12-17) *	500.00	At transplanting	60
	Ammonium nitrate (26-0-0) *	230.77	At the first mechanical weeding	60

Table 1. *Cont.*

Treatment	Fertilizer	Dose (kg ha^{-1})	Distribution Period	N (Units ha^{-1})
(4) Mix-Conventional a	Animal pellet (3-0-0) *	1333.33	At transplanting	40
	Ammonium nitrate (26-0-0) *	307.69	At the first mechanical weeding	80
(5) Mix-Conventional b	Animal pellet (3-0-0) *	2666.66	At transplanting	80
	Ammonium nitrate (26-0-0) *	153.84	At the first mechanical weeding	40
(6) Organic + Agroecological practices	Organic vegetable amendment "Vegand" (4.1,4-0) *	3000.00	At transplanting	120

* N-P_2O_5-K_2O, respectively.

As can be deduced from Table 1, the fertilizers were applied considering the normal uptake of organic cauliflower (120 units per ha) in the tested area, but they were distributed differently. Indeed, in our two-year experimental trial, three different soils with three different histories were used with the final aim of comparing consolidated agronomical practices. Specifically, we included organic soils (treatments 1 and 2) that had been under organic management for 20 years, agroecological soil (treatment 6) that had been under agroecological practices for 20 years, and conventional soils (treatments 3, 4, and 5), which had been conventionally managed for 20 years. Treatment 6 constitutes an important reference and is integrated in this research because it represents the condition of a virtuous and environmentally friendly farmer.

The cauliflowers were transplanted in 2018 on the 22 August and in 2019 on the 21 August, with a spacing of 0.52 m per row and 0.80 m between rows (24.000 plants ha^{-1}). Mechanical weeding was carried out on the 11 September 2018 and 17 September 2019, respectively. Plant defense was applied using, for all the treatments, the same commercial formulations, as follows: Airone (copper oxychloride + copper hydroxide) and Cuproxat (copper sulphate) as fungicides; Xentari (Bacillus thuringensis) and Spinosad (Spinosina A + Spinosina B) as insecticides; and Slux (ferric phosphate) to control snails. The harvest took place on the 11 and 2 March in 2019 and 2020, respectively.

Soil sampling (n. 6 theses x n. 4 replicates = n. 24 samples) was carried out every year at the time of cauliflower harvesting for each elementary plot. Four sub-samples of about 250 g each were taken from both within and between the plant rows to obtain a better sampling uniformity, and then they were pooled in only one sample. A share of this amount (about 350 g) was sent to the laboratory. Stable isotope ratio ($^{15}N/^{14}N$ and $^{34}S/^{32}S$) analyses were performed on freeze-dried soil samples.

Leaf sampling (n. 6 theses x n. 4 replicates = n. 24 samples) was carried out every year at the time of cauliflower harvesting for each elementary plot. The leaves were sampled by taking two plants and three leaves/plant: one outer leaf, one intermediate leaf, and one young leaf near the inflorescence. Stable isotope ratio ($^{15}N/^{14}N$, $^{13}C/^{12}C$, $^{34}S/^{32}S$ and $^{2}H/^{1}H$, $^{18}O/^{16}O$) analyses were performed on freeze-dried leaves samples.

A sample of corymb (n. 6 theses x n. 4 replicates = n. 24 samples) for each elementary plot was collected every year at the time of cauliflower harvesting. The physicochemical parameters (fresh weight, total soluble solids, total acidity, cut resistance, CIE L*, a*, b* color indices, head height, and head diameter) were determined on fresh corymb samples, while the vitamin C content, total phenolic content, in vitro antioxidant activity, total and inorganic N, and stable isotope ratio ($^{15}N/^{14}N$ $^{13}C/^{12}C$ and $^{34}S/^{32}S$) analyses were performed on freeze-dried corymb samples.

All the samples (soil, leaves, and corymb) were analyzed, with four experimental replicates for each treatment for each of the two years of experimentation. Each experimental replicate was analyzed in triplicate.

2.2. Physicochemical Parameters

The fresh cauliflower quality parameters were assessed on a representative sample of four heads collected at commercial maturity from each of the six different agronomic treatments. Corymb weight, head height, and head diameter were measured on the edible white corymb of each sample, and average values (mean of four measurements) were obtained for each of the six treatments. The total acidity (TA) and pH were determined by titration with a 0.1 M sodium hydroxide solution using an automatic titrator (Mettler Toledo (Greifensee, Switzerland), DL 25) and expressed as a % of citric acid. The total soluble solids (TSS) were determined using a digital refractometer (ATAGO (Minato-ku, Tokyo, Japan), RX-5000) and expressed as °Brix. The cut resistance was measured using a texture analyzer (Zwick/Roell (Ulm, Germany), DO-FB0.5 TS model 2002), with the maximum resistance to cutting measured on two opposites of the edible white corymb of each sample. Finally, the color analysis was evaluated as flesh CIE L*, a*, b* values using a chroma meter (Minolta (Chiyoda-ku, Tokyo, Japan), CR-300).

2.3. Ascorbic Acid Content

The ascorbic acid (Vitamin C) content was determined on a 500 mg freeze-dried corymb sample extracted with a 3.0% metaphosphoric acid solution. The sample was centrifuged at 5000 rpm for 20 min and filtered using a 0.45 µm cartridge prior to HPLC injection. The data were expressed on a dry weight basis. The HPLC analysis conditions were as follows: the column was a Luna (Phenomenex, Torrance, CA, USA) 250 mm × 4.6 mm i.d. 5 µm column, and the mobile phase was a 0.02 M phosphoric acid solution, run at a flow rate of 1.0 mL/min. Detection was performed at 260 nm.

2.4. In Vitro Antioxidant Activity

In order to evaluate the antioxidant activity of the freeze-dried corymb samples, two methods were used: ORAC and FCR assays. The extraction was carried out by placing 100 mg of freeze-dried corymb samples with 100 mL of an ethanol–water solution 80:20 (v/v) at 1% citric acid in flasks fitted with a cap and kept stirring at 120 rpm for 24 h at a temperature of 25 °C. At the end of the 24 h period, the hydroalcoholic extract was filtered and set aside; the residue was stirred again with another 100 mL aliquot of solvent, under the same experimental conditions; after 24 h, this extract was filtered and added to the first one. The combined extracts were then subjected to distillation under vacuum in a rotavapor lab system to remove the residual ethanol. The extracts were then employed for further determinations of antioxidant activity.

2.4.1. Folin–Ciocalteu Reagent (FCR)

The FCR colorimetric method is based on an electron-transfer (ET) reaction with the oxidant as an indicator of the reaction's endpoint, and it is a colorimetric method usually used to determine total phenolics. It was applied as previously described [32], with some modifications, to evaluate the antioxidant activity of the cauliflower samples. Appropriately diluted concentrated aqueous extract (1:50, v/v) samples (1 mL) were mixed with 5 mL of FCR commercial reagent (previously diluted with water 1:10 v/v) and 4 mL of a 7.5% sodium carbonate solution. The mixture was stirred for 2 h at room temperature while avoiding strong light exposure. The absorbance of the resulting blue solution was measured spectrophotometrically at 740 nm, and the concentration of total phenolics was expressed as (±) gallic acid equivalents (mg g^{-1} DW).

2.4.2. Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay represents a hydrogen atom transfer (HAT) reaction mechanism and measures the antioxidant scavenging activity against peroxyl radicals induced by AAPH. It was used as described by Cao [33], adapted and modified. Briefly, the measurements were performed on a Wallac 1420 Victor III 96-well plate reader (Perkin Elmer, Shelton, DC, USA) equipped with fluorescence filters (excitation 485 nm, emission 535 nm). Fluorescein

(116 nM) was the target molecule for the free radical attack from AAPH (153 mM) as the peroxyl radical generator. The reaction was conducted at 37 °C, at pH 7.0, with Trolox (1 µM) as the control standard and 75 mM of phosphate buffer (pH 7.0) as the blank. All the solutions were freshly prepared prior to the analysis. Concentrated aqueous extracts were diluted with a phosphate buffer (1:50, *v*/*v*) prior to their analysis, and the results were reported as µmoles of Trolox equivalents per g DW.

2.5. Total and Inorganic N

The total N (mg Kg^{-1}) was measured after dry matter digestion of the freeze-dried corymb samples in concentrated sulfuric acid, distillation with NaOH (10 mol L^{-1}) and boric acid 2%, and titration with HCl (0.1 mol L^{-1}), according to Kjeldahl's procedure, using the AutoKjeldahl Unit K-370 (Buchi, Flawil, Switzerland) [34].

Nitrate and ammonium were determined as follows: 25.0 g of ground and freeze-dried sample was transferred into a 50.0 mL glass beaker, added to 25 mL of distilled water, homogenized by stirring in a water bath, at a temperature of +80 °C for 20 min. Once cooled, distilled water was added up to the marked line, shaken until homogeneously mixed, and filtered. A total of 5.0 mL of the first filtrate was discarded, and the following filtrate was collected. The filtrate obtained was used for the determination of inorganic nitrate and ammonium; the concentration assay was performed using a continuous flow colorimetry autoanalyzer (Flow SyS Systea, Anagni, Italy).

2.6. Stable Isotope Ratios' Analysis

All the corymb samples were subjected to $^{13}C/^{12}C$, $^{15}N/^{14}N$, and $^{34}S/^{32}S$ analyses using an isotope ratio mass spectrometer (Delta plus XP Thermo Finnigan, Bremen, Germany) equipped with an elemental analyzer (EA Flash 1112 Thermo Finnigan). The values of the isotopic ratios were expressed in δ ‰, calculated against working in-house standards (mainly atropine), and calibrated against international reference materials including NBS-22 oil (IAEA) for $\delta^{13}C$ and glycine (USGS 64) for $\delta^{15}N$ measurement. Hair USGS 42 ($\delta^{34}S$ = +7.84 ± 0.25 ‰), USGS 43 ($\delta^{34}S$ = +10.46 ± 0.22‰), and barium sulphates IAEA-SO-5 and NBS 127 (IAEA) were used for $^{34}S/^{32}S$ calibration.

The freeze-dried samples of the leaves were weighed in tin (2.5 mg for $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$) or silver capsules (0.25 mg for $^{2}H/^{1}H$ and $^{18}O/^{16}O$) on a microbalance. The dried soil samples and the freeze-dried fertilizers were weighed in tin capsules (10 mg) for $^{15}N/^{14}N$ and $^{34}S/^{32}S$ analyses. The comparative equilibration method was used for the analysis of $^{2}H/^{1}H$. The samples and standards were left in the laboratory's air moisture for a minimum of 96 h and then placed in a desiccator with P_2O_5 under N atmosphere. The $^{13}C/^{12}C$, $^{15}N/^{14}N$, and $^{34}S/^{32}S$ isotope ratios were all measured using an isotope ratio mass spectrometer (Vision, Elementar, Langenselbold, Germany) via a continuous flow inlet system to a Vario Isotope Cube Elemental Analyser (Elementar, Germany). Both the $^{2}H/^{1}H$ and $^{18}O/^{16}O$ isotope ratios were analyzed using an isotope ratio mass spectrometer (Finnigan DELTA XP, Thermo Scientific, Bremen, Germany) after complete pyrolysis in an elemental analyzer (Finningan DELTA TC/EA, high-temperature conversion elemental analyzer, Thermo Scientific).

As per the IUPAC protocol, the isotopic values were expressed as a delta with respect to the international standard V-PDB (Vienna-Pee Dee Belemnite) for $\delta(^{13}C)$, V-SMOW (Vienna-Standard Mean Ocean Water) for $\delta(^{2}H)$ and $\delta(^{18}O)$, Air (atmospheric N_2) for $\delta(^{15}N)$, and V-CDT (Vienna-Canyon Diablo Troilite) for $\delta(^{34}S)$, following Equation (1):

$$\delta_{ref}({}^{i}E/{}^{j}E, sample) = \left[\frac{R({}^{i}E/{}^{j}E,\ sample)}{R({}^{i}E/{}^{j}E,\ ref)}\right] - 1 \quad (1)$$

where *ref* is the international measurement standard; *sample* is the analyzed sample; and ${}^{i}E/{}^{j}E$ is the isotope ratio between heavier and lighter isotopes [35]. The delta values are

multiplied by 1000 and commonly expressed in units "per mil" (‰) or, according to the International System of Units (SI), as a "milliurey" (mUr) [36].

The isotopic values were calculated against two standards through the creation of a linear equation. Two internal working standards calibrated using international reference materials were used for $^{13}C/^{12}C$ and $^{15}N/^{14}N$. As for $^{13}C/^{12}C$, fuel oil NBS-22 ($\delta(^{13}C) = -30.03 \pm 0.05$‰), sucrose IAEA-CH-6 ($\delta(^{13}C) = -10.45 \pm 0.03$‰) (IAEA-International Atomic Energy Agency, Vienna, Austria), and L-glutamic acid USGS 40 ($\delta(^{13}C) = -26.39 \pm 0.04$‰) (U.S. Geological Survey, Reston, VA, USA) were employed. As for $^{15}N/^{14}N$, L-glutamic acid USGS 40 ($\delta(^{15}N) = -4.52 \pm 0.06$‰) (U.S. Geological Survey, Reston, VA, USA) and potassium nitrate IAEA-NO3 ($\delta(^{15}N) = +4.7 \pm 0.2$‰) were adopted. Keratins CBS (Caribou Hoof Standard $\delta(^{2}H) = -157 \pm 2$‰ and $\delta(^{18}O) = +3.8 \pm 0.1$‰) and KHS (Kudu Horn Standard, $\delta(^{2}H) = -35 \pm 1$‰ and $\delta(^{18}O) = +20.3 \pm 0.2$‰) from the U.S. Geological Survey were used to normalize the $^{18}O/^{16}O$ and $^{2}H/^{1}H$ values. Hair USGS 42 ($\delta^{34}S = +7.84 \pm 0.25$‰), USGS 43 ($\delta^{34}S = +10.46 \pm 0.22$‰), barium sulphates IAEA-SO-5 and NBS 127 (IAEA) were applied for $^{34}S/^{32}S$ calibration.

Each reference material was measured in duplicate at the start and end of each daily group of samples (each sample was also analyzed in duplicate). A control material was also included in the analyses of each group of samples to check the measurements' validity. The accepted maximum standard deviations of repeatability were 0.3‰ for $\delta(^{13}C)$, $\delta(^{15}N)$, and $\delta(^{34}S)$, 0.5‰ for $\delta(^{18}O)$, and 4‰ for $\delta(^{2}H)$.

2.7. Statistical and Chemometric Multivariate Analysis

Statistical analysis was conducted using the STATSOFT 6.0 software (Statsoft Italia srl, Vigonza, Italy). Significant differences were evaluated by means of a variance analysis (ANOVA), and means separation was conducted using Tukey's post hoc test. For all the parameters, the analysis of variance was carried out considering the "years" and "fertilization treatments" as fixed factors. Since the factor "years" and the interaction between the two factors were not significant ($p > 0.05$) for most of the parameters investigated, we herein present, for each of the determined parameters, the average values for each treatment for the two years of experimentation. To evaluate the real efficiency of discrimination between the six different treatments, based on a qualitative and stable isotopic analysis, a multivariate statistical method, such as linear discriminant analysis (LDA), was applied using the IBM SPSS Statistics 20 software (IBM corporation; Armonk, NY, USA) using the within-class covariance matrix as the classification model.

3. Results and Discussion

3.1. Physicochemical and Qualitative Cauliflower Parameters

Table 2 reports the results of the physicochemical and qualitative cauliflower parameters determined on the fresh corymb samples collected at commercial maturity on each of the two subsequent years of trial. The results are expressed as a mean of the two years of cultivation.

Table 2. Physicochemical and qualitative cauliflower parameters. The data reported are average values ± standard errors, relating to four experimental replicates for each treatment for two years of experimentation (N = 8). Different letters indicate statistically significant differences ($p \leq 0.05$).

Treatment	Corymb Weight (g FW)	pH	Total Acidity (% Citric Acid)	Total Soluble Solids (° Brix)	Corymb Head Height (cm)	Corymb Head Diameter (cm)	L*	A*	B*	Cut Resistance (N)
Organic	981.23 ± 59.57 *ab*	6.42 ± 0.05	0.21 ± 0.01	6.62 ± 0.21 *b*	10.91 ± 0.57	15.41 ± 0.87	73.35 ± 2.14	−0.41 ± 0.06	19.88 ± 1.00	50.14 ± 0.02
Mix-Organic	967.78 ± 38.17 *ab*	6.35 ± 0.05	0.21 ± 0.00	6.65 ± 0.13 *b*	10.91 ± 048	15.34 ± 0.48	69.86 ± 1.81	−0.43 ± 0.07	18.61 ± 0.39	50.10 ± 0.01
Conventional	931.96 ± 69.92 *ab*	6.42 ± 0.04	0.20 ± 0.01	7.56 ± 0.33 *a*	10.34 ± 0.63	14.25 ± 0.63	71.23 ± 1.03	−0.26 ± 0.03	18.34 ± 0.43	50.18 ± 0.05
Mix-Conv.—a	863.44 ± 59.57 *b*	6.47 ± 0.04	0.21 ± 0.01	7.26 ± 0.19 *ab*	11.84 ± 0.35	15.31 ± 0.35	74.02 ± 1.38	−0.27 ± 0.13	19.21 ± 1.22	50.29 ± 0.14
Mix-Conv.—b	881.90 ± 36.34 *ab*	6.49 ± 0.03	0.20 ± 0.01	7.04 ± 0.25 *ab*	10.19 ± 0.46	14.09 ± 0.46	75.52 ± 1.18	−0.10 ± 0.06	20.31 ± 0.14	50.22 ± 0.04
Organic + AEP	1141.61 ± 80.08 *a*	6.43 ± 0.04	0.20 ± 0.01	6.62 ± 0.25 *b*	12.06 ± 0.76	15.97 ± 0.76	73.98 ± 1.41	−0.36 ± 0.03	19.61 ± 0.59	49.42 ± 0.64

The results pointed out that the main physicochemical parameters determined on the fresh edible corymb of the cauliflowers collected from the six different plots did not show

any significant difference. Indeed, the product characteristics in terms of carpometric, color, and consistency properties showed no differences with respect to the applied fertilization practices, showing that the type of N applied to the soil does not produce any effect on the progress of the physiological ripening of the corymb nor on the total acidity or pH of the corymb. Conversely, significant differences in the corymb's fresh weight and total soluble solids content were found. The fresh corymb weight showed significantly ($p \leq 0.05$) higher values in the "Organic + Agroecological practices", showing a positive effect of the agroecological practices, as found in other studies [37–39]. In particular, the introduction of these techniques significantly increased this parameter by 17.15 and 27.64% compared to the mean of the organic and conventional treatments, respectively. Even if not significant, this treatment also reached the highest absolute values of both corymb height and diameter with respect to the other treatments. The positive effect of the agroecological treatment on the average fresh weight of the corymbs can also be related to the fact that, in the MOVE LTE, soil fertility has been improved (from 1.1% to 1.8%) thanks to the use of rotating cover crops, such as velvet vetch, wheat, and horseradish, which have been grown every year since 2001. In this plot, the soil fertility of the field has improved, starting from 1.1% of organic matter at the beginning of field conversion to 1.8% of the actual organic matter content, as the result of long-term sustainable procedures. This final amount is the same in recent years, as a result of the equilibrium of organic agriculture productions. This condition should represent farms that have been growing organically for several years. The total soluble solids significantly ($p \leq 0.05$) accumulated and showed higher values in the conventional treatment with respect to the other fertilization treatments. This behavior may be due to the synthetic N being immediately available to the plant, which generates an enhancement in the maturation rate, producing a faster increase in the total soluble solids content in this treatment [40,41] despite the equal fertilization units applied in all the fertilization treatments. The total soluble solids resulted to be inversely correlated with corymb fresh weight (r= -0.2390; $p \leq 0.05$).

3.2. Ascorbic Acid Content

The results of the HPLC determination of the ascorbic acid content in the freeze-dried corymb samples are reported in Table 3. The average values for each treatment were significantly different ($p \leq 0.05$), with higher values in the "Organic" and, to a lesser extent, in the "Mix-Organic" treatments. Lower values were recorded in the three conventional treatments ("Conventional", "Mix-Conventional—a", and "Mix-Conventional—b") and in the "Organic + Agroecological practices" one. As far as the highest content of this bioactive compound in the two organic treatments is concerned, there are several research studies supporting this experimental evidence. In the last two decades, it has been hypothesized that the accumulation of ascorbic acid in organic products may be linked to the N deficiency in these products with respect to the immediately available synthetic N fertilizers applied to conventional practices [12,13]. Furthermore, it has been theorized that organic plants, following a higher pathogen pressure due to the lack of pesticide application, change their metabolism, inducing the biosynthesis of secondary metabolites as an endogenous mechanism for plant defense [14]. More recently, it has been suggested that organic management induces an adaptive metabolism that turns into a continuous supply of carbon skeletons for the biosynthetic demands of organic plants [42]. Our results are in accordance with these previous studies, confirming that organic cauliflower accumulates higher levels of ascorbic acid with respect to different conventional treatments. The use of horseradish as an agroecological service crop combined to the use of an organic fertilization treatment of vegetable origin in the "Organic + Agroecological practices" plot have influenced the accumulation of ascorbic acid in the final product, with lower values being recorded with respect to the "Organic" treatment which was fertilized using organic animal pellets. Indeed, other authors have recently reported that the application of different cover crops may differentially affect the ascorbic acid content of the main crop [43]. Specifically, the authors of the aforementioned study found that different vegetable species employed as

cover crops, in a monoculture or intercropped, differentially affected the ascorbic acid content and other bromatological characteristics of green and sweet corn.

Table 3. Ascorbic acid content in cauliflower. The data reported are average values ± standard errors, relating to four experimental replicates for each treatment for two years of experimentation (N = 8). Different letters indicate statistically significant differences ($p \leq 0.05$).

Treatments	Ascorbic Acid (mg g^{-1} DW)
Organic	8.16 ± 0.37 *a*
Mix-Organic	6.94 ± 0.37 *ab*
Conventional	5.52 ± 0.71 *abc*
Mix-Conventional—a	5.77 ± 067 *abc*
Mix-Conventional—b	3.56 ± 0.75 *c*
Organic + Agroecological practices	3.88 ± 0.62 *bc*

3.3. In Vitro Antioxidant Activity

A Folin–Ciocalteu reagent and ORAC assays were used to test the in vitro antioxidant capacity of the cauliflower samples collected from the six different treatment plots in which different agronomic practices were applied. Figure 1 shows the results of the two assays. The total polyphenols content resulted to be significantly higher ($p \leq 0.01$) in the "Organic" treatment (53.02 ± 0.99 mg g^{-1} DW)compared to the three conventional ones ("Conventional", "Mix-Conventional—a", and "Mix-Conventional—b") and the "Organic + Agroecological practices" treatment, while the "Mix-Organic" treatment recorded intermediate average values. Once again, as for the ascorbic acid values, the accumulation of polyphenolic metabolites in the fully organic treatment can be ascribed to the metabolism adaptation of organic crops that, differently to the conventional ones, activates a cascade supply of carbon skeletons as a response to the endogenous biosynthetic demands and needs of the crops' mechanisms of defense. Indeed, a recent study demonstrated that organic soil management promoted salicylic acid build-up, which was not associated with the lower N content of organic plants but depended on alterations in the soil microbial communities associated with long-term organic management [44]. It has also been suggested that further studies are needed to better understand how organic management promotes beneficial soil microbial populations and which are the biochemical mechanisms underlying organic plants' accumulation of polyphenolic compounds, a phenomenon which is induced in such plantations [45].

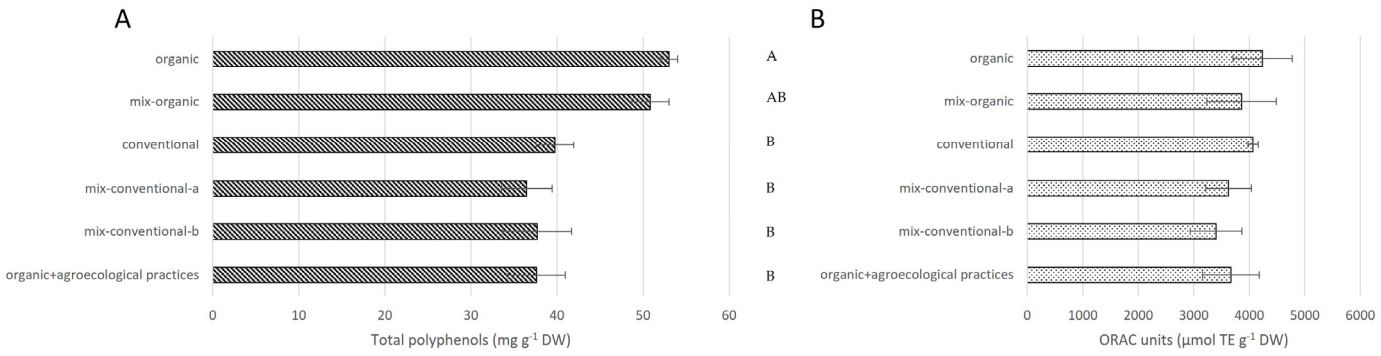

Figure 1. Total polyphenol content (**A**) and ORAC units (**B**) in cauliflower (four experimental replicates for each treatment for two years of experimentation; N = 8) ± standard errors. Different letters indicate statistically significant differences ($p \leq 0.01$).

The ORAC units represent an internationally recognized measure of the antioxidant capacity of a food. It is widely recognized that foods higher in ORAC units are thought to be richer in antioxidants, helping in the protection of humans against the detrimental effects

of the free radicals associated with aging and disease. Even though there is not an officially recommended daily intake of antioxidants in terms of ORAC units, it is widely agreed that the daily intake of fruits and veggies providing at least 3000–5000 ORAC units is recommended to assure an efficient antioxidant protection and counteract the physiological action of free radicals [46]. The results of our study showed that no statistically significant difference was recorded in terms of ORAC units among the different treatments, while the relevant biological value of the cauliflower samples was highlighted. As described above, in the "Material and Methods" section, an ORAC assay is based on a HAT mechanism, while FCR in based on an ET reaction. Therefore, even though similar trends in the in vitro antioxidant activity determined using the two different methods were recorded for the cauliflower samples collected from the six different treatments, it is reasonable that, due to the different chemical mechanism on which the two assays are based, statistically significant differences were evidenced only in the total polyphenols content. This may be putatively linked, in cauliflower, to the higher reactivity of antioxidant compounds that exert their antioxidant potential by exchanging an electron atom with respect to that of other compounds acting as hydrogen donors/acceptors.

3.4. Total and Inorganic Nitrogen

The average concentrations of total N and total inorganic, nitric, and ammoniacal N in the cauliflowers collected from the six different agronomic plots are reported in Table 4. The results showed no statistical difference among the six different agronomic treatments, showing that the different N source (organic or conventional or mixed) produced no imbalance in the N metabolism of the cauliflower plants during their ripening, also taking into account that, within each treatment, fertilization equally provided 120 kg N ha^{-1}. This result suggests that it is possible to substitute totally or partially mineral fertilizers with organic ones, thus promoting the sustainability of organic production, as found in a different environment with other organic materials on the same crop [47]. The nitrate levels in the cauliflower samples in our study are in line with those previously reported for the Chambord F1 cauliflower cultivar [48] and for cauliflower seedlings [49], showing no effect of the six different fertilization strategies applied on the preferential accumulation of these compounds.

Table 4. Total N and total inorganic, nitric, and ammoniacal N concentrations in cauliflower. The data reported are average values ± standard errors, relating to four experimental replicates for each treatment for two years of experimentation (N = 8).

Treatment	Total Nitrogen (%)	Total Inorganic Nitrogen (mg kg^{-1} DW)	NO_3^- (mg kg^{-1} DW)	NH_4 (mg kg^{-1} DW)
Organic	3.77 ± 0.20	241.69 ± 18.44	27.68 ± 5.64	214.00 ± 13.55
Mix-Organic	3.85 ± 0.12	290.84 ± 26.33	29.81 ± 3.89	261.03 ± 22.64
Conventional	3.27 ± 0.20	224.00 ± 24.47	28.59 ± 3.32	195.41 ± 21.25
Mix-Conventional—a	3.19 ± 0.26	252.84 ± 28.35	22.35 ± 3.86	230.49 ± 24.75
Mix-Conventional—b	3.02 ± 024	225.45 ± 27.73	23.74 ± 4.42	201.71 ± 23.51
Organic + Agroecological practices	3.11 ± 014	202.03 ± 13.71	16.81 ± 3.95	185.21 ± 13.62

Given that cauliflower grows well at 18–25° and, consequently, takes advantage of days with good sunlight, it is presumable that the low concentration of nitrates found in the cauliflowers coming from the six different fertilization strategies may be attributable, in addition to the cultivation techniques, also to the position and exposure of the field, which favored good radiation and helped the reduction of nitrates. Indeed, it is widely known that the nitrate levels in plants are influenced by several external factors, such as soil, fertilization, air, and also harvest and storage [50]. Furthermore, as mentioned above, it is known that, in response to light, the enzyme nitrate reductase (localized in the chloroplast of leaves) and the protein ferredoxin (localized in nitrite reductase) increase their activity,

thus favoring the reduction of nitrates with the formation of NH_4^+ [51]. In addition, the presence of light increases the availability of carbohydrates and organic acids (which are produced via photosynthesis), which accumulate in the vacuole as an alternative to nitrate. Therefore, in the presence of good sunlight, there is a greater production of organic N by plants and a subsequent release of nitrate accumulation.

3.5. Stable Isotope Ratios' Analysis

3.5.1. $^{15}N/^{14}N$ and $^{34}S/^{32}S$ Analyses of Fertilizers

The fertilizers applied in this study and collected in the two years of experimentation presented values of $\delta(^{15}N)$ (Table 5) perfectly fitting in the ranges of the values previously reported by Bateman and Kelly [8] for the several types of fertilizers they analyzed. In particular, the $\delta(^{15}N)$ values of the synthetic fertilizers fall within a narrow range close to 0‰ and lying between −2.2 and 1.0‰. On the other hand, as expected, the fertilizers coming from animal manure presented a value of $\delta(^{15}N)$ noticeably higher, around 8‰, while the plant-derived fertilizers presented higher values ranging between 2.2 and 3.5‰.

Table 5. $\delta(^{15}N)$ values determined in the fertilizer samples.

$\delta(^{15}N)$ (‰ vs. AIR)	Type of Fertilizer
7.9	Organic animal pellet (1st year)
2.2	Vegand (1st year)
1.0	YaraMila Blustar (1st year)
−0.2	Ammonium nitrate (1st year)
8.1	Organic animal pellet (2nd year)
3.5	Vegand (2nd year)
0.7	YaraMila Blustar (2nd year)
−2.2	Ammonium nitrate (2nd year)

3.5.2. $^{15}N/^{14}N$ and $^{34}S/^{32}S$ Analyses of Soil

In Figure 2, the box plots of the $\delta(^{15}N)$ and $\delta(^{34}S)$ values in the soil of the six treatments considered in this study are presented. The impact of conventional versus organic and mixed products was statistically evaluated. No statistically significant differences ($p \leq 0.01$) were found according to the fertilization strategies for $\delta(^{34}S)$. This result confirms previous studies [52] that reported, despite some effects related to the type of fertilizer applied, to some extent, a higher sensibility of this parameter to geographical origin rather than agricultural system. Indeed, the homogeneity of the ranges of variability in the tested soil from the six treatments is a confirmation that it this homogeneity is related to the geological characteristics of the soil, the microbial activity, and the proximity to the sea [52]. On the other hand, the ANOVA test found statistically significant differences ($p \leq 0.01$) according to the agricultural cultivation practices for $\delta(^{15}N)$. In particular, the highest value was recorded in the agroecological treatment, which was characterized by a soil with a longer organic history, while lower values were recorded for the completely conventional treatments and intermediate values for the mixed ones. The $\delta(^{15}N)$ trend is a reflection of the nature of the fertilization or farming systems that have been implemented on the soil during the last few years, which left an impression on the soil. Therefore, "historically" organic soil presented statistically higher values than the soil with a conventional background. Indeed, the fertilizers synthetically obtained via the Haber–Bosch process contained N derived from the air, and, therefore, they presented $\delta(^{15}N)$ values around 0‰, whereas the organic fertilizers obtained from animal waste or vegetable matter had higher values. These results were due to the preferential atmospheric ^{14}N losses during organic fertilizers' storage and the isotopic fractionation occurring during biological processes, and they confirmed the results reported in other studies [8]. It is also clear that the agroecological management of the soil led to completely outstanding values of $\delta(^{15}N)$, distinguishable also from those of soil under organic management.

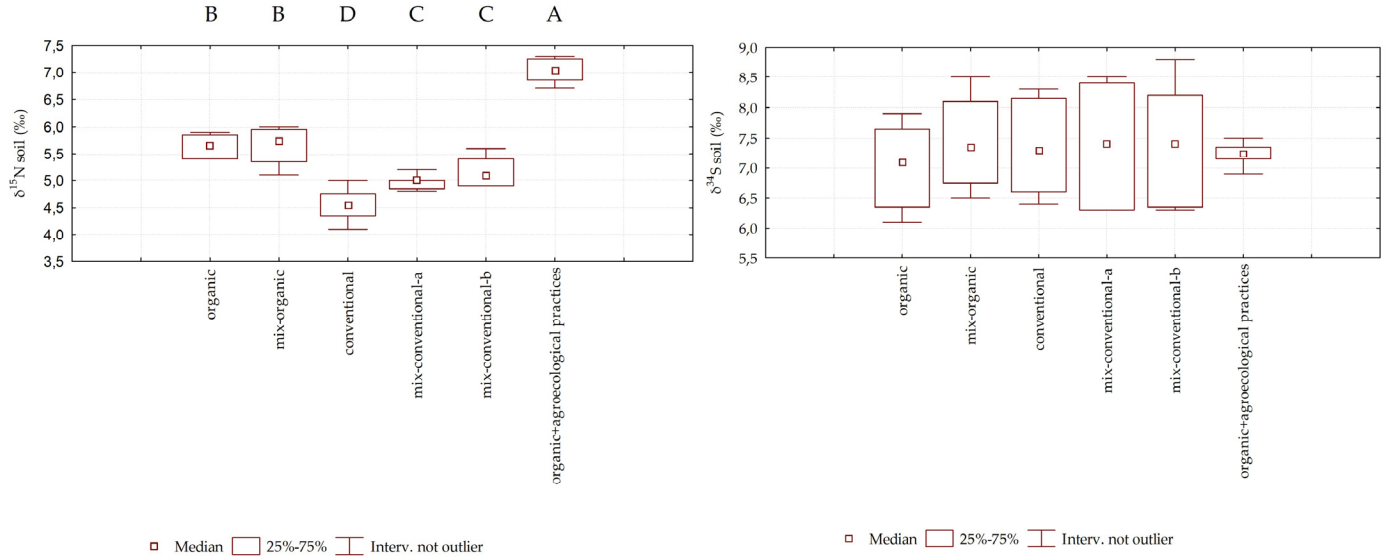

Figure 2. Box plot of $^{34}S/^{32}S$ [$\delta(^{34}S)$] and $^{15}N/^{14}N$ [$\delta(^{15}N)$] in the soil samples (four experimental replicates for each treatment for two years of experimentation; N = 8). Different letters indicate statistically significant differences ($p \leq 0.01$).

3.5.3. $^{15}N/^{14}N$, $^{13}C/^{12}C$, $^{34}S/^{32}S$ and $^{2}H/^{1}H$, and $^{18}O/^{16}O$ Analyses of Leaves

In Figures 3 and 4, the distribution of the values of $\delta(^{2}H)$, $\delta(^{13}C)$, $\delta(^{15}N)$, $\delta(^{18}O)$, and $\delta(^{34}S)$ of the cauliflower leaves are presented according to the fertilization strategies used. No significant effects ($p \leq 0.01$) of the fertilization conditions were observed both for $\delta(^{18}O)$ and $\delta(^{2}H)$. Indeed, these two parameters are frequently used for the geographical authentication of food products [24] as they are more linked to the geo-climatic characteristics of the place of growth of the plants than to the fertilization practices employed. No significant differences were also found for $\delta(^{13}C)$, which ranged for all the samples in a very narrow range of values around −29.0‰, characteristic for plants with a C3 photosynthetic cycle. With respect to the $\delta(^{34}S)$ of the leaves, a clear pattern related to the type of fertilizer applied was not shown. A very high dispersion of the data was recorded, as far as $\delta(^{13}C)$ and $\delta(^{34}S)$ were concerned, highlighting that these parameters may not contribute significantly to the discrimination between different fertilization strategies. The pattern of $\delta(^{15}N)$ was completely clear and related to the fertilization practices applied during plant growth, with a higher value in the leaves from organic management, a lower value for the conventional treatment, and intermediate values for the mixed treatments, essentially measuring higher values in the treatments grown on a soil "historically" under organic management, probably due to a buffer effect of the soil's original isotopic composition.

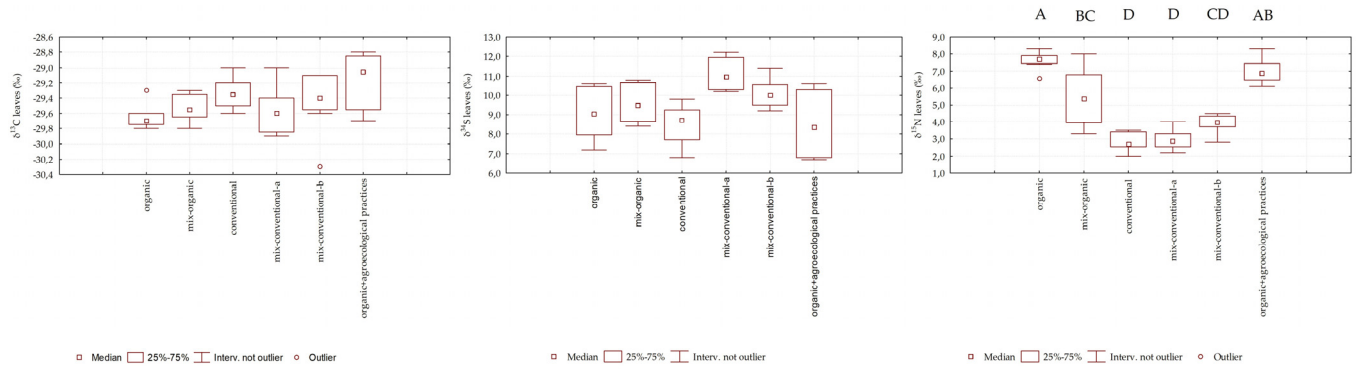

Figure 3. Box plot of $^{13}C/^{12}C$ [$\delta(^{13}C)$], $^{34}S/^{32}S$ [$\delta(^{34}S)$], and $^{15}N/^{14}N$ [$\delta(^{15}N)$] in cauliflower leaves (four experimental replicates for each treatment for two years of experimentation; N = 8). Different letters indicate statistically significant differences ($p \leq 0.01$).

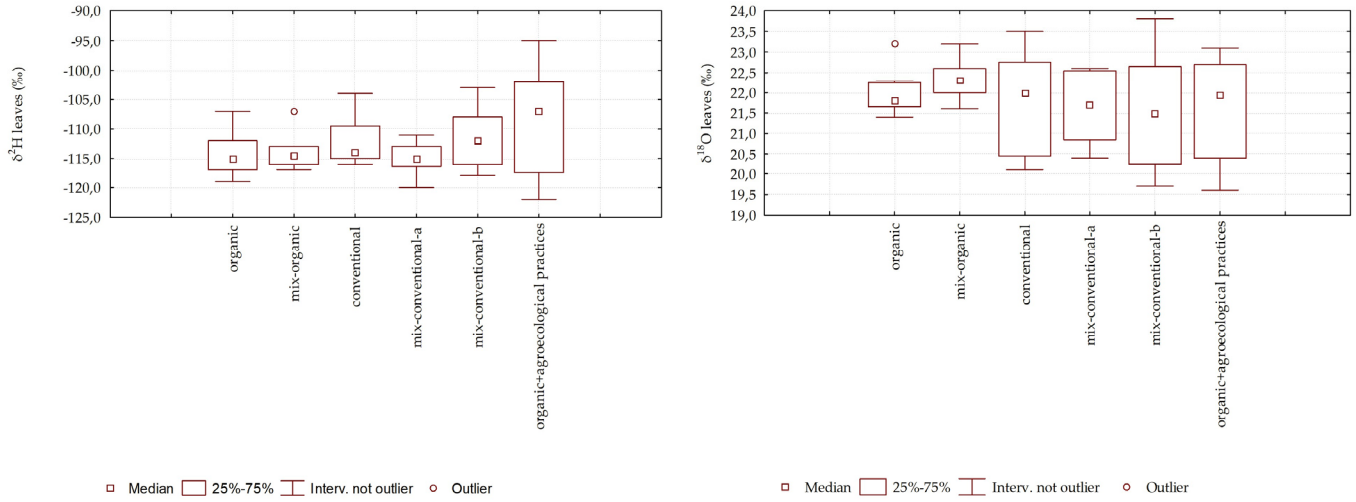

Figure 4. Box plot of $^{2}H/^{1}H$ [$\delta(^{2}H)$] and $^{18}O/^{16}O$ [$\delta(^{18}O)$] in cauliflower leaves (four experimental replicates for each treatment for two years of experimentation; N = 8).

3.5.4. $^{15}N/^{14}N$ $^{13}C/^{12}C$ and $^{34}S/^{32}S$ Analyses of Corymb Samples

The results of the stable isotope ratio analysis of $^{13}C/^{12}C$, $^{34}S/^{32}S$, and $^{15}N/^{14}N$ in the corymb samples collected from the six different plots are shown in the box and whisker plots reported in Figure 5. The $\delta(^{13}C)$ and $\delta(^{34}S)$ values showed no statistically significant difference with respect to the different fertilization practices applied in the six plots. The main origin of isotopic fractionation for C in plants is linked to the metabolic pathway employed for carbon dioxide fixation and subsequent sugar biosynthesis [53]. It is known that C3 plants follow the Calvin cycle for carbon dioxide fixation, and, with respect to C4 plants, they have lower $^{13}C/^{12}C$ ratios since the heavier isotope is kinetically disadvantaged in this metabolic pathway. Because C4 plants exist only at lower latitudes where warmer climatic conditions are present, there is a decreasing gradient of $^{13}C/^{12}C$ from the equator to the poles. This behavior has been widely exploited for the identification of the geographical origin of a crop and/or its derived products [53] but not for the discrimination of a product's cultivation practice, confirming our results. Also, limited application of the use of $\delta(^{13}C)$ for the discrimination of the employed fertilization practice has been reported, as $\delta(^{13}C)$ may be affected by an animal's diet, producing differences in organic amendments of animal origin [14], but our study showed no relevant significant differences. In our study, no effect on the $\delta(^{34}S)$ values was recorded. It is known that $\delta(^{34}S)$ provides useful information mainly regarding geographical origin, as it is related to the soil and the sulphate fertilizer employed [54]. Indeed, it is known that no fractionation occurs during sulfate's reduction to organic sulfur in plants [54], and our results confirmed this evidence. On the other hand, the $\delta(^{15}N)$ values showed significant statistical differences among the six treatments, with the higher values being recorded for the "Organic" and "Organic + Agroecological practices" treatments. The $\delta(^{15}N)$ values showed the lowest values for the "Conventional" and "Mix-Conventional—a" treatments, while intermediate values were recorded for the "Mix-Conventional—b" and the "Mix-Organic" treatments, demonstrating increasing values when increasing percentages of organic fertilizers were employed. Compared to air, N has about the same isotopic composition throughout the world ($\delta(^{15}N_{AIR})$ = 0‰) [55]. The natural processes of nitrification, denitrification, ammonification, and other biological processes that occur in the soil produce a significant isotopic fractionation that depends on the climatic characteristics of the area and, above all, on the soil and N fertilizer used. Therefore, it is recognized that the N isotope ratio is not linked to the latitude but to the agronomic practices used [56]. The production of synthetic N fertilizers does not cause fractionations with respect to the N isotope ratio of air; therefore, synthetic fertilizers have isotope ratios close to 0‰. The production and maturation processes of organic fertilizers, composts, and amendments, however, involve a significant fractionation due to the prefer-

ential volatilization of the ammonium containing the lighter N isotope, with a consequent increase in the N isotope ratio. N uptake by plants in soil causes only a small fractionation and, hence, only slightly alters the isotopic composition of the residual fertilizer or soil organic matter. Therefore, the differences in the N isotope ratios of synthetic or organic fertilizers can be used as indicators of the agricultural regime employed [8]. The results of our study confirmed this evidence. The $\delta(^{15}N)$ values showed significant differences among the six treatments. Indeed, the "Organic" and "Organic + Agroecological practices" treatments showed significantly higher average values, equal to 7.09 ± 0.13 and 7.50 ± 0.22‰, respectively, when compared to other treatments. As indicated in the "Materials and Methods" section, the "Organic + Agroecological practices" treatment included the use of a cover crop (in particular horseradish) combined to fully organic fertilization with an organic fertilizer of vegetable origin. In such a way, it was possible to evaluate the effect of the application of agroecological practices to the $\delta(^{15}N)$ values of open-field-cultivated organic cauliflowers. Our results showed no influence of the applied cover crop on the $\delta(^{15}N)$ values, which showed similar values to those of the "Organic" treatment crops, which had been fertilized with organic animal pellet. This behavior may be explained by the fact that horseradish is not a N-fixing plant; therefore, it does not influence the N isotope ratio of the soil. Moreover, the cauliflowers grown using this treatment were able to benefit not only from the N of vegetable origin coming from the VEGAND organic fertilizer but also from the N derived from the mineralization of the organic substance in the soil of the plot. This soil, as detailed in the "Material and Methods" section, had a history of over twenty years of organic management, which included, in addition to the use of three different cover crops, the application of organic fertilizer of animal origin. The lowest $\delta(^{15}N)$ values were recorded for the "Conventional" and "Mix-Conventional—a" treatments, which resulted to be equal to 2.51 ± 0.65 and 3.49 ± 0.43‰, respectively. As expected, mineral N fertilization produced a significant decrease in the N isotope ratio of the cultivated crop. The "Mix-Conventional—b" (2/3 organic animal pellet + 1/3 ammonium nitrate on a soil which had always been managed using conventional techniques) and "Mix-Organic" (1/3 organic animal pellet + 2/3 ammonium nitrate on a soil which had not undergone any chemical treatment for several years) treatments, which accounted for mixed-fertilization strategies, showed intermediate values equal to 4.00 ± 0.53 and 6.23 ± 0.48‰, thus demonstrating the influence of the supply of increasing percentages of synthetic fertilizers in organic plots and vice versa. As there are not scientifically recognized confidence ranges for the $\delta(^{15}N)$ values in organic crops, the determination of their value still remains an analytical and very powerful support when checking for organic authenticity. The $\delta(^{15}N)$ values herein reported for the mixed fertilization strategies, i.e., "Mix-Conventional—a", "Mix-Conventional—b", and "Mix-Organic", highlighted that considering the N isotopic ratio to be an effective and univocal biomarker of the cultivation method could putatively result in a misclassification of a horticultural crop if relevant percentages of mineral/organic fertilizers are simultaneously employed in organic/conventional practices. Incidentally, our results demonstrate that the more synthetic N is applied, the lower the $\delta(^{15}N)$ of the cauliflower samples is.

Based on our results, the stable isotope ratio analysis of $^{13}C/^{12}C$ and $^{34}S/^{32}S$ showed no relevant discriminating influences with respect to the different fertilization practices applied in the six plots. The $^{15}N/^{14}N$ ratio values showed to be effective as indicators of the applied agricultural practices for the fully organic (treatments 1 and 6) and conventional (treatment 3) treatments, while the values reported for the mixed fertilization strategies (treatments 2, 4, and 5) showed that this parameter, considered alone, cannot be considered a biomarker of the cultivation method as misclassification could occur.

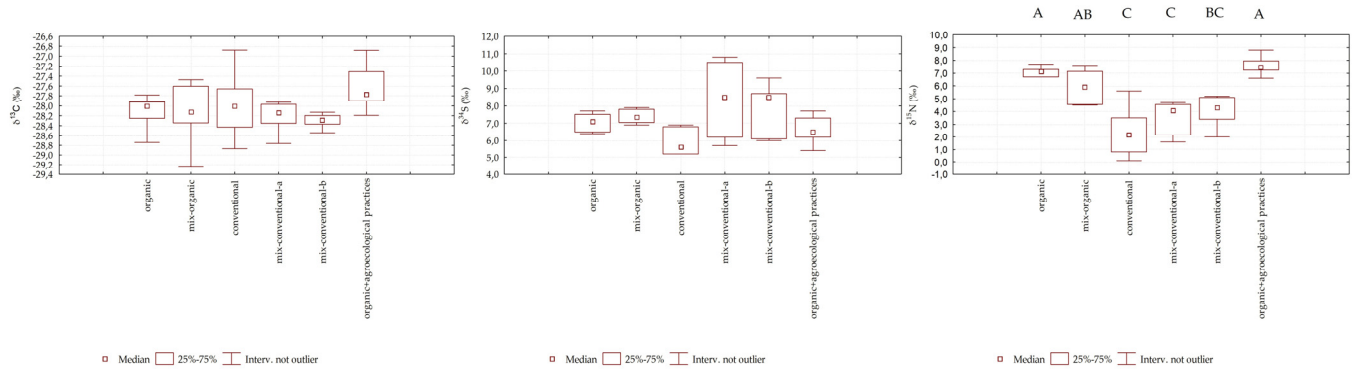

Figure 5. Box plot of $^{13}C/^{12}C$ [$\delta(^{13}C)$], $^{34}S/^{32}S$ [$\delta(^{34}S)$], and $^{15}N/^{14}N$ [$\delta(^{15}N)$] in cauliflower (four experimental replicates for each treatment for two years of experimentation; N = 8). Different letters indicate statistically significant differences ($p \leq 0.01$).

3.6. Chemometric Multivariate Analysis

An integrated multivariate statistical approach was used to classify the cauliflower production of six different agronomic treatments with the aim of evaluating if and to what extent products obtained using different agronomic practices could be correctly classified and distinguished from one another based on their physicochemical, nutritional, and isotopic profile, jointly combined. A linear discriminant analysis (LDA) was employed as a multivariate statistical method to verify the feasibility of assigning a sample to one of the previously defined groups (the six different agronomic treatments) on the basis of its compositional similarity to the groups themselves. The LDA analysis allowed us to obtain a chemical interpretation of the system and provided a graphical representation of the experimental results by transforming the original variables in new variables called standardized canonical discriminant functions, which were function of the original data. The first five canonical discriminant functions, which together explained 100% of the cumulative variance of the system, were used to apply the classification model. In Table 6, the eigenvalues of the five canonical discriminant functions and the relative percentages of variance, cumulated variance, and canonical correlations are reported. As far as the structure matrix of the system is concerned, the within-group correlations between the discriminant variables and the standardized canonical discriminant functions showed that the $\delta(^{15}N)$ of the soil and corymb samples had the highest absolute correlations with the first canonical discriminant function, equal to 0.459 and 0.248, respectively. The standardized coefficients of the canonical discriminant functions associated with each variable used in the analysis are reported in Table 7. They constitute the absolute contribution (weight) that each original variable made in the creation of the canonical discriminant functions. Taking into consideration the fact that the first canonical discriminant function is an expression of the larger part of the variance in the data (67.4%), Table 6 shows that the variables that contributed the most to the classification model were the total polyphenols (2.220), the $\delta^{15}N$ of soil (0.921), the TSS (−0.717), and the $\delta^{15}N$ of corymb (0.643). Thus, our results highlighted that the most relevant differences among the six treatments were to be ascribed to the total soluble solids, the differential accumulation of secondary metabolites belonging to the polyphenolic class, and the N isotopic ratio. Figure 6 shows the graphical representation of the implemented LDA classification model obtained using the first two canonical discriminant functions as the cartesian coordinates. The samples belonging to the six different agronomic treatments are displayed as they were classified by the LDA. With respect to the first function, all six different treatments are discriminated from one another, and only a partial overlapping of the data from treatments 1 and 2 can be detected. Indeed, the results of the classification showed that 100% of the original cases were correctly classified. It may be concluded that the multivariate approach employed was able to correctly classify the belonging of each sample to its group. Treatments 2, 4, and 5, which represent mixed fertilization practices, and treatment 6, which represent green manure use,

were introduced in the experimental design to test the reliability of the proposed system also in samples obtained by applying agronomic practices usually employed in commercial fields. To the best of our knowledge, this is the first time that a classification model has shown that, in cases of mixed conditions (green manure use, supply of organic fertilizers in conventional practice, or vice versa), only a model of multivariate analysis, including $\delta^{15}N$ and other quality parameters, can contribute to a reliable discrimination between organic and conventional cauliflower products.

Table 6. Eigenvalues of the canonical discriminant functions generated by LDA.

Function	Eigenvalue	% Variance	% Cumulated Variance	Canonical Correlation
1	40.767	67.4	67.4	0.988
2	10.431	17.3	84.7	0.955
3	5.512	9.1	93.8	0.920
4	2.338	3.9	97.7	0.837
5	1.407	2.3	100.0	0.765

Table 7. Standardized coefficients of the canonical discriminant functions.

	Function				
	1	**2**	**3**	**4**	**5**
Corymb_weight	0.060	0.478	0.360	0.264	−0.045
pH	−0.515	−0.417	0.297	−0.112	0.626
Total_acidity	−0.157	−0.473	−0.812	0.245	−0.276
TSS	−0.717	1.037	0.679	0.414	−0.108
Ascorbic_acid	−0.519	0.054	0.040	−0.467	−0.584
Total_polyphenols	2.220	1.054	0.679	0.416	−0.166
Total_nitrogen	−0.500	−0.182	0.716	−0.431	−1.677
Total_inorganic_nitrogen	0.564	1.783	−1.872	3.361	1.792
NO_3	−1.116	0.760	1.272	−0.383	0.801
$\delta^{15}N$_corymb	0.643	−0.134	0.811	−0.247	−0.777
$\delta^{13}C$_corymb	0.362	−0.365	0.219	−0.173	−0.242
delta_34S_corymb	0.126	−0.140	−0.963	−0.446	0.239
$\delta^{15}N$_soil	0.921	−0.523	0.273	0.374	−0.213
$\delta^{34}S$_soil	−0.532	1.922	1.310	3.811	0.705
$\delta^{15}N$_leaves	0.510	0.746	−1.257	−0.037	1.349
$\delta^{13}C$_leaves	−0.499	0.473	1.343	0.129	0.372
$\delta^{34}S$_leaves	0.129	−0.997	−0.181	0.370	0.108
$\delta^{2}H$_leaves	0.572	−0.447	0.157	0.275	−0.049
$\delta^{18}O$_leaves	−0.317	0.981	−0.579	−0.653	0.501
Corymb_head_height	−0.365	−2.545	−1.593	−1.099	−0.789
Corymb_head_diameter	0.343	2.787	1.077	1.180	0.081

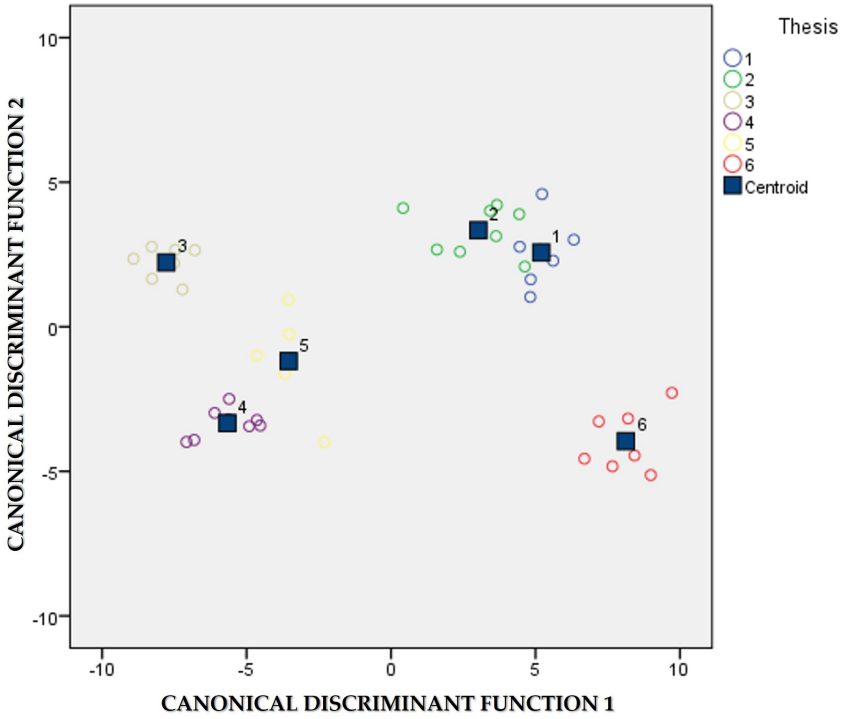

Figure 6. Graphical representation of the first two canonical discriminant functions. Treatments: (1) "Organic"; (2) "Mix-Organic"; (3) "Conventional"; (4) "Mix-Conventional—a"; (5) "Mix-Conventional—b"; and (6) "Organic + Agroecological practices".

4. Conclusions

As innovative tools for nitrogen fertilization traceability in organic farming products are severely needed to help stakeholders, retailers, and policy makers counteract food frauds in the organic food sector, we proposed the application of a multivariate-integrated approach to an open-field cultivation case study for cauliflower crops. Diverse agronomic treatments were adopted and compared, including conventional, organic, and mixed treatments at different % of mineral fertilizers, also comprising the application of agroecological service crops, which may ensure more sustainable cultivation techniques compared to organic and conventional traditional treatments. The results of this study highlighted that detected differences in the isotopic and qualitative parameters of cauliflowers and soil, which, in our study, were mainly affected by the combination of the type of fertilizer applied during plant growth, may not be used alone as biomarkers of the cultivation method. Specifically, based on the results of the applied multivariate LDA, it was demonstrated that only a multivariate approach is able to correctly classify 100% of the grouped cases. Moreover, it was shown that the variables that contributed the most to the classification model were the total polyphenols, the $\delta^{15}N$ of the soil, the TSS, and the $\delta^{15}N$ of the corymbs, confirming that the N isotopic ratio and the content of secondary metabolites belonging to the polyphenolic class differentially accumulate in organic vs. conventional products. Taking into consideration the fact that TSS and polyphenolic levels may undergo alteration during transportation, storage, and distribution, it is advisable that samples are analyzed while fresh. If this is not possible, it is advisable that control bodies keep frozen or freeze-dried control samples. These results are very promising and suggest that further application of the proposed approach should be carried out on other horticultural crops, with the aim of validating the evidence achieved so far.

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editing, M.A., L.B., G.C., F.M., N.T., B.T. and S.F.; visualization, M.A., L.B., G.C., F.M., N.T., B.T. and S.F.; supervision, S.F.; project administration, S.F.; funding acquisition, S.F. All authors have read and agreed to the published version of the manuscript.

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