

Metabolite Changes in *Vitis Vinifera* (L.) cv Garganega Leaves and Berries After 501 Biodynamic Treatment

Mario Malagoli (✉ mario.malagoli@unipd.it)

University of Padova - Viale dell'Università, 16, 35020 - Legnaro PD

Stefania Sut

University of Padova - Viale dell'Università, 16, 35020 - Legnaro PD

Gourav Kumar

University of Padova - via Marzolo 5 35121 - Padova -

Stefano Dall'Acqua

University of Padova - via Marzolo 5 35121 - Padova -

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Abstract

Background

There is a need for new approaches in agriculture to improve safety of final products as well as to increase environmental acceptability. In this paper, the biodynamic preparation 501 (horn-silica) was sprayed on *Vitis vinifera* (L.) cv Garganega plants in two vineyards located in Veneto region, North-East Italy. Leaf samples were collected on the day of 501-treatment and 11 days later, and berries were sampled at harvest time. Leaves and berries samples were analysed combining targeted and untargeted measurements related to primary metabolism (pigment, element and amino-acid contents) and to secondary metabolism. Chlorophyll content in leaves, and amino-acid and element (C,N,S) analysis in berries were combined with untargeted UPLC-QTOF metabolomics.

Results

The discriminant compounds related to the 501-treatment were annotated on the basis of accurate MS and fragmentation and were identified as secondary metabolites, namely phenolic constituents belonging to the shikimate pathway. The level of most of the identified compounds increased in plants treated with 501 preparation.

Conclusions

Results highlight the prominent value of the metabolomic approach to elucidate the role of the 501 applications on grapevine secondary metabolism.

Background

Conventional agriculture relies upon intense utilization of chemical inputs. Visible consequences are the reduction of soil organic matter content, the rise of soil desertification, the chemical contamination of soil and surface and underground water. To counteract this alarming trend, actions for sustainable approaches in agriculture have been implemented in the last years [1]. Organic farming, agro-ecology, permaculture and biodynamic farming are the most diffuse alternative systems to conventional agriculture [2].

Biodynamic agriculture was developed by Rudolf Steiner in 1924 [3], who proposed the use of preparations (numbered from 500 to 508) to be applied on soil, over crops, and in compost pile, with the main aim to improve soil vitality and plant growth and health.

Biodynamic farming emphasizes biodiversity, takes into consideration the influence of celestial bodies and promotes the concept of farm as an organism. Although biodynamics is internationally recognized as a valuable farming system, studies on the effects of the preparations on soil and plants are limited.

Viticulture is an important branch in the agricultural sector of many countries, including Italy. The spread of several chemical compounds to control pathogens, parasites and weeds in vineyards has raised concerns among consumers, producers and policy-makers about the possible negative impacts on the ecosystem and human health [4]. Alternative approaches for a more sustainable grapevine cultivation are then desirable. In the last decades, several winegrowers approached the biodynamic method [5], as a concrete way to encounter the increasing demand of consumers on the quality and sustainability of grape and wine production.

Following Steiner's indications, the biodynamic preparation 500 consists on fresh cow manure placed inside a cow horn buried in the top soil during the autumn-winter months, and preparation 501 is pure quartz powder left for about 5 months in a cow horn buried in the top-soil. The spreading of 500 on soil and 501 over the canopy in the vineyards was found to enhance the vegetative-reproductive balance of grape plants [6]. Positive effects on the application of preparations on the soil microbiology and fertility [7, 8], and on the plant resistance to biotic and abiotic stresses, were also evidenced in different grape varieties [9, 10]. Picone *et al.* found changes in grape berry metabolome due to biodynamic management, likely related to physiological response of the plants treated with preparations [11]. The application of horn silica preparation (501) was noticed to positively affect the quality of grapes and wines [12, 13].

Variations on different biochemical compounds, such as total polyphenols, anthocyanins polymeric pigments and phytocompounds, like catechin, cyanidin-3-glucoside, flavonoid, esters, protocatechuic acid, resveratrol and quercetin were reported in berries and wines after the spreading of preparations [10]. To validate the effect of biodynamic preparations, various approaches have been used, including conventional measurements used in agronomic trials, but also spectral techniques as nuclear magnetic resonance for metabolic confirmation [11, 14].

The analyses on sugars, amino-acids and some carboxylic acids content in berry juice are routinely performed using enzymatic methods or some high pressure liquid chromatography approaches [15]. More advanced techniques, combining the Ultra Performance Liquid Chromatography with the High-resolution Mass Spectrometry, offer comprehensive and unbiased approaches of metabolomics. Larger amount of information can be obtained about different groups of compounds in grape tissues, juices and wines than the traditional targeted analysis [15].

The aim of this study was to assess possible metabolic changes induced by the application of preparation 501 in plants of *Vitis vinifera* cv Garganega. A number of targeted analysis, namely leaf chlorophyll content, amino-acid and element (C,N,S) content in berries, were combined with untargeted UPLC-QTOF metabolomic analysis performed both in leaves and berries.

Materials And Methods

Experimental site and sampling

The experiment was performed in two vineyards, Roncaie (R) and Paiello (P), located in Gambellara, Vicenza county, North East of Italy (45°27'44"N 11°20'00"E), during the period June-September 2018. Plants of *Vitis vinifera* L. cv Garganega (20–30 years old) with “tendone” as training system are biodynamically managed since 2005 with the use of preparations.

In each vineyard, a plot of six rows with 25 vines per each row was selected. Three rows were left as control (no 501 treatment) and three rows were spread with 501. The two inner rows (one with and one without 501 treatment) were considered as buffer rows and only the remaining rows were used for sampling and data collection. The preparation 501 was obtained from the certified biodynamic preparations dealer Le madri (Rolo, Italy). In the early morning of May 10th, 3 g of 501 were dissolved in 50 L of rain water and mixed for 1 hour, in order to have sufficient solution to treat 1 ha canopy. The solution was manually spread over the grape canopy in both the vineyards. Concomitantly, the control plants were sprayed with the same volume of rain water only.

Leaves samples were collected 6 hours after the 501 treatment (sampling 1, May 10, 2018) and 11 days later (sampling 2, May 21, 2018) from four randomly chosen plants in each row. The fourth leaf in the stem was detached, immediately transferred in a plastic bag and frozen. A total of 16 leaves per sampling time was collected from both control and treated plants in each plot.

At harvest time, about 100 berries/plant were sampled from the same plants where leaves were collected and transferred in plastic bag and frozen.

A schematic description of the sample timing and analyses is reported in Fig. 1

Soil analysis

Five soil samples (30 cm² and 30 cm depth) were randomly collected 3 days before the starting of the experiment from the selected plots in each of the two vineyards. Individual samples were air dried, crushed and passed through a 2 mm sieve. The samples from the same plot were then bulked to obtain homogenous samples.

Soil pH was measured potentiometrically on 1:2.5 soil/water extracts. Total carbon and total nitrogen were determined by dry combustion in a CNS Vario Macro elemental analyser (Elementar, Hanau, Germany). Organic carbon was obtained by subtraction of inorganic fraction. Exchangeable bases, including calcium (Ca), magnesium (Mg), potassium (K), and sodium (Na), were extracted using barium chloride and their concentration was determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) on a SPECTRO CIROS (Spectro Analytical Instruments, Kleve, Germany).

Leaf pigment measurement

Frozen leaves were cut in small pieces exactly weighted (200mg), crashed in mortar with 5 mL ethanol 95%(v/v), and then transferred in volumetric flask with the addition of ethanol to the final volume of 10 mL. Flasks were stored at 4°C in dark condition for 24 hours. The supernatant was filtered and used to

measure UV absorbance at 665, 649, and 470nm with Elios α spectrophotometer (Thermo). The concentration of chlorophylls *a* and *b* was then calculated [16].

Metabolomics analysis

Sampled leaves were cut in small pieces exactly weighted (200mg) and extracted using Ultrasound Assisted Extraction with 10 mL methanol 90% (v/v) for 10 minutes. Extracts were centrifuged at 13000 rpm for 10 minutes and used for analysis. Berries were crushed in a mortar, and the obtained juice was centrifuged, filtered 0.45 μ and used for analysis. Each biological replicate was analysed in triplicate.

To obtain a metabolomic profile of the leaves and berries, a UPLC-HR-MS full-scan method was used. Waters H-Class UPLC system equipped with a Waters Xevo G2 Q-TOF mass spectrometer was employed. The detector was equipped with an electrospray (ESI) ionisation source and was operating in negative ion mode. The sampling cone voltage was adjusted at 40 V, the source offset at 80 V. The capillary voltage was adjusted to 1500 V. The nebulizer gas used was N₂ at a flow rate of 800 L h⁻¹. The desolvation temperature was 450°C. The mass accuracy and reproducibility were maintained by infusing lockmass (leucine–enkephalin, *m/z* 556.2771) thorough Lockspray at a flow rate of 20 μ L min⁻¹. Centroided data were collected for each sample in the mass range 50–1200 Da, and the *m/z* value of all acquired spectra was automatically corrected during acquisition based on lockmass. An Agilent XDB C-8 column (2.1 mm x 150 mm, 3.5 μ m) was used as stationary phase. The mobile phase was composed of solvent A (acetonitrile with 0.1% formic acid) and solvent B (water with 0.1% formic acid). Linear gradients of solvents A and B were used, as follows: 0 min, 10% A; 10 min, 85% A; 11 min, 100% A; 12 min, 10% A; 12.5 min, 10% A. The flow rate was 300 μ L min⁻¹ and the injection volume was 1 μ L. Pool samples were prepared mixing equal amount of all samples. To control instrument performance during run sequence of samples were randomised and each 6 injection pool samples were injected.

Data extraction and analysis: centroided and integrated chromatographic mass data were processed by MarkerLynx Applications Manager version 4.1 (Waters) to generate a multivariate data matrix. A method for data deconvolution, alignment and peak detection was created and the data were subsequently elaborated by the software. The parameters used were retention-time range 1.00–19.00 min, mass range 100–1200 Da, mass tolerance 0.01 Da, noise elimination level was set to 6.00, minimum intensity was set to 12% of base peak intensity, maximum masses per RT was set to 6 and, finally, RT tolerance was set at 0.01 min. A list of the ion intensities of each peak detected was generated, using retention time and the *m/z* data pairs as the identifier for each ion. The resulting three-dimensional matrix contains arbitrarily assigned peak index (retention time-*m/z* pairs), sample names (observations), and ion intensity information (variables). As first step, the matrix was composed of 11060 mass x variables and to reduce the number of components, we proceed with the exclusion of the variables having more than 30% of missing data in the different groups under investigation. The obtained matrix was log-transformed and normalized by Median Fold Change normalization and mean centred. The obtained matrix was then elaborated using SIMCA (Umetrics) software and PCA and PLS-DA models were generated. Compounds describing the different groups were selected. To assess the non-presence of overfitting data Y-axis

intercepts after permutation test were calculated. Time × mass variables contributing to group separation were selected on the basis of their variable importance on projection (VIP) value. Only variables bearing VIP values > 11 were considered significant in differentiating the groups and were subsequently putatively identified using accurate mass value, fragmentation pathway generated using MS^e function of the Q-TOF mass spectrometer. The tables in the results section report the variation (%) calculated from the average area of annotated metabolite in the treated samples compared to the average area of the same metabolite in control samples.

CNS content in berries

Berries were dried completely in oven at 80°C and then crushed to make fine powder. Ten mg of powdered samples were used to measure the carbon, nitrogen and sulfur content by vario micro cube instrument run at CHNS analyzer (Elementar Vario ELIII).

Analysis of amino-acids

Berry juice (0.3 mL) was added to 5 mL HCl (0.5 M) for 10 minutes at room temperature. Solution were centrifuged and used for analysis. Standard solutions were prepared weighting exact amount of each amino-acid in HCl solution (0.5 M) in four different concentrations from 10 µg mL⁻¹ to 1 µg mL⁻¹. For analysis, an Agilent Z-HILIC Column (3 x 100 mm, 4 micron) was used as stationary phase, and eluents were acetonitrile, (A) and water 0.5% formic acid (B). A gradient program was used as follows: 0 min, 95% A; 1 min, 95%A; 11 min, 70%A; 14 min, 40% A, 14.5 min, 95%A then isocratic for 5 minutes. Flow rate was 0.450 mL min⁻¹. Each amino-acid transition was optimized with corresponding standard solution. MS/MS parameter was as reported in previous work [17].

Statistical analysis

Data on amino-acids, chlorophylls, CNS, secondary metabolites were subjected to a one-way analysis of variance (ANOVA). All the experiments were performed in triplicate.

Related to the metabolomics analysis the MS data deriving from the Q-TOF were transformed in matrix that was imported into Simca 12 software (Umetrix, Sweden). Unsupervised Principal Component (PCA) and supervised Orthogonal Partial Least Square Discriminant (OPLS-DA) analyses were performed using centering and pareto scaling. The unsupervised PCA was carried out to observe homogeneous sample clusters that were used as Y classes in the supervised PLS-DA. As final output, compounds that are related to the cluster separation in OPLS-DA were identified. The statistical analyses were validated by performing permutation test (150 permutations); CV-ANOVA ($p < 0.05$); t -test ($p < 0.05$) for a cluster characterizing molecule.

Results

Soil characterization

Soil in Paele (P) vineyard is sandy-clay loam while the Roncaie (R) vineyard soil is clay loam.

Both soils are mild alkaline with similar organic carbon content, but Roncaie soil has a higher level of inorganic carbon. P soil exhibited higher amount of potassium and magnesium than R soil. The opposite was measured for calcium and sodium content (Additional Table 1).

Changes induced in leaf tissues by 501

The preparation 501 is commonly sprayed over the canopy in the early morning, in absence of rainfall and wind. Consequently, the leaves are the organs of the plants mostly exposed to the treatment. The effect of 501 on leaves was assessed by measuring the content of chlorophyll a and b, as markers of impact on the leaf photosynthetic apparatus, and by an untargeted UPLC-QTOF-MS method followed by multivariate data analysis to estimate metabolite variations.

Leaves pigment measurement

The content of chlorophyll a and chlorophyll b in the leaves of control plants resulted higher than in 501-treated plants of Paele vineyard, and the opposite was found in the leaves of plants in Roncaie vineyard, but with no statistically significant difference (Table 1).

Untargeted-metabolomic analysis

To assess any possible effect of the 501 distribution, metabolomic analysis was performed in leaves of *Vitis vinifera* L. cv Garganega plants at two different times, one immediately after 501 application (T1), and 11 days later (T2). Finally, berries of treated and control plants were analysed at harvest time. The two vineyards, Paele and Roncaie, were chosen to assess any possible variation due to location.

Table 1
Leaf content of chlorophyll a (Chla) and chlorophyll b (Chlb) (mg g^{-1} fresh weight). P- Paele; R- Roncaie; C- control; T- treated with 501; 1-sampling date May 10; 2-sampling date May 21.

	Chla	Chlb
PC1	0.84 ± 0.23	0.49 ± 0.23
PT1	0.78 ± 0.27	0.39 ± 0.20
PC2	1.03 ± 0.24	0.46 ± 0.17
PT2	0.78 ± 0.27	0.39 ± 0.24
RC1	0.71 ± 0.25	0.24 ± 0.08
RT1	0.78 ± 0.29	0.24 ± 0.11
RC2	0.51 ± 0.23	0.19 ± 0.02
RT2	0.69 ± 0.11	0.34 ± 0.22
Data are means of n = 6 samples ± standard deviation		

UPLC-QTOF-MS metabolomics was performed in negative ion mode and allowed the identification of metabolites on the basis of the retention time, accurate m/z value, MS^e fragmentation pattern. The datasets were elaborated first using Principal Component Analysis (PCA) to observe any possible clusterization of the samples. The stability of chromatographic system and MS spectrometer was assessed, as shown by the “pool” samples that are well clusterized in the centre of the plot for the samples of both the vineyards (Additional Fig. 1, 2). To highlight the differences between sampling times 1 and 2, data from control and treated plants were also elaborated using the supervised method by Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA). Each multivariate model will lead to a graphical representation of treated vs control samples for each vineyard. Detailed elaboration and results are described in the following chapters.

Metabolomic analysis of leaves in the Paiele Vineyards

The data related to the Paiele leaf samples are illustrated in Additional Fig. 3. Green and Blue colours indicate treated samples at time 1 and 2, respectively, while red and yellow colours represent control samples at time 1 and 2, respectively. The samples can be grouped on the basis of time sampling, as highlighted by the red and blue ellipsoid. No clusterization of control (C) versus treated (T) samples is observed. This indicates that metabolomic changes can be ascribed to the time of sampling rather than to the 501 treatment. For a more detailed analysis, a multivariate data elaboration (OPLS-DA) was performed considering sampling time 1 and 2 separately.

The OPLS-DA models highlight changes of leaf metabolites (control vs treated), immediately after the biodynamic treatment (sampling time 1) (Fig. 2A) and 11 days later (sampling time 2) (Fig. 2B).

The treated groups in both the sampling times mostly occupy the -x,-y part of the graph showing a treated vs control grouping. The changes in leaves composition were identified thanks to UPLC-MS data as phenolic compounds, mostly ascribable to flavonoids, stilbenoids and hydroxycinnamic acid derivatives. Identification of the compounds that mostly described differences between treated and control samples, and their relative amount are reported in Table 2.

Table 2

Differential metabolite changes in leaves of Paielle plants treated with preparation 501 (PT) compared to control (PC) at both sampling times. Compounds presenting VIP > 11 were selected, putative identification was obtained considering HR-MS spectra as well as fragmentation obtained by MS^e approach.

Retention time	Var ID (m/z)	PT1 vs PC1	PT2 vs PC2	Identification
4.05	463.0889	+ 1.0%	+ 1.5%	Quercetin-glucoside*
4.16	593.1519	+ 0.2%	+3.2% §	Keampferol-rutinoside
4.12	289.0704	+ 0.7%	+ 2.7%	Epi-Catechin
5.38	227.1283	+ 1.0%	+1.5% §	Resveratrol*
4.56	477.0686	+ 3.0%	+16.0% §	Quercetin-glucuronide
5.62	507.2453	+12.0% §	+0.5% §	Dicaffeoyl hexose
4.76	609.1472	+ 1.5%	+ 32.0% §	Rutin*
4.79	447.0938	+ 5.0%	+ 11.0% §	Kaempferol-glucoside
4.82	461.0727	+ 3.0%	+ 11.0% §	Methoxy kaempferol glucoside
4.86	301.0352	+ 1.5%	+ 1.0%	Quercetin*
(*) When available, reference compounds were used to confirm findings. § indicates statistical difference $p < 0.05$.				

At sampling time 1, 6 hours after the 501-treatment, a general increase of the selected phenolic secondary metabolites is observed in leaves of 501-treated plants compared to controls, but only the content of dicaffeoyl hexose augmented significantly. At sampling time 2, 11 days after the 501 distribution, significant variation for seven of the selected phenolic metabolites can be noticed. The results indicate that 501-treatment mainly induced the increase in the amount of kaempferol and quercetin derivatives, as well as in resveratrol, epicatechin and caffeoyl ester with hexoside.

Metabolomic analysis of leaves in Roncaie Vineyards

The data related to the Roncaie leaves samples are shown in Additional Fig. 4. Light green and light blue colours are associated to treated samples at time 1 and 2 respectively, while brown and orange colours represent control samples at time 1 and 2, respectively. As for Paielle samples, the PCA revealed a grouping due to time sampling as indicated by Blue and Red ellipsoids. No clusterization of control (C) versus treated (T) samples is observed. Even in Roncaie vineyard, the most intense changes can be ascribed more to the sampling time than to the 501-treatment. The multivariate data elaboration (OPLS-DA) was then performed considering sampling time 1 and 2 separately.

The OPLS-DA models show the changes in leaf metabolites (control vs treated) occurred soon after the biodynamic treatment (sampling time 1) (Fig. 3A) and 11 days later (sampling time 2) (Fig. 3B).

Table 3

Differential metabolite changes in the leaves of Roncaie plants treated with preparation 501 (RT) compared to controls (RC), at both sampling times. Metabolites presenting VIP > 11 were selected, putative identification was obtained considering HR-MS spectra as well as fragmentation obtained by MS^e approach.

Retention time	Var ID (m/z)	RT1 vs RC1	RT2 vs RC2	Identification
2.40	311.0395	+ 2,6%	+ 301,6% §	Caftaric acid*
2.40	179.0335	+ 2.2%	+ 298.2% §	Caffeic acid*
4.83	415.1960	+ 5.7%	+ 29.7%	Ethyl 7-epi-12-hydroxyjasmonate glucoside
4.02	431.1911	+ 3.0%	+25.0% §	S-furanopetasitin
4.12	289.0704	-1.5% §	-12.7% §	Epi-Catechin*
5.38	227.0717	-2.5% §	-60.2% §	Resveratrol*
4.79	447.0924	+ 1.1%	+1.1% §	Kaempferol-glucoside
4.76	609.1460	+ 2.0%	+3.2% §	Rutin*
3.61	289.0705	-5.0% §	-12.0% §	Catechin*
(*) When available, reference compounds were used to confirm findings. § indicates statistical difference $p < 0.05$.				

The treated groups mostly occupy the + x part of the graphs in both the sampling times, showing grouping of treated vs control samples. The UPLC-MS data revealed that phenolic compounds level changed. Identification of the compounds that mostly highlight the differences between treated and control group and their relative amount are reported in Table 3.

The leaves of 501-treated plants displayed a variation of phenolic compounds compared to controls at both sampling dates. Epicatechin, resveratrol and catechin, significantly decreased compared to control at both sampling times. The application of 501 boosted the level of caftaric acid (+ 301.6) and caffeic acid (+ 298.2) at sampling time 2, and significant increase was recorded for Kaempferol-glucoside, S-furanopetasitin and Rutin. The augmented level of the two metabolites, ethyl 7-epi-12-hydroxyjasmonate glucoside and S-furanopetasitin, not belonging to the class of polyphenols, was also recorded.

3. Assessing changes induced by 501-treatment in berries

Limited information is available up to now to the possible influence of biodynamic treatment with 501 on berries of grapevine. Some targeted measurements, namely the elemental composition (C, N, S) and the amino-acid content, and untargeted UPLC-QTOF-MS metabolomics analysis were performed on berries to assess the effects of the 501 application.

3.1 C, N, S and amino-acid content

The treatment with preparation 501 did not vary significantly the content of sulfur, carbon and nitrogen in the berries (Additional Table 2). In 501-treated berries the amount of sulfur (+ 7% in PT and 14% in RT) and nitrogen (+ 16% in PT and + 9% in RT) was slightly higher than controls.

The berry content of free amino acids was also investigated. Aspartic acid and proline were the two most abundant in berries of both vineyards. Significant increased levels of cysteine (+ 49.9%), methionine (+ 100%), phenyl alanine (+ 24.9%), and decreased amounts of proline (-21.1%), arginine (-26.2%) and serine (-21.0%) were observed in 501-treated fruits compared to control, in Paiele vineyard (Table 4).

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Significant higher amount of cysteine (+ 133.3%), arginine (+ 36.0%), tryptophan (+ 67.8%) and lower concentration of isoleucine (-23.8%) threonine (-8.4%) tyrosine (-9.3%) were measured compared to control in Roncaie berries (Table 4). Overall, the distribution of 501 induced distinct, although limited, variations in the berry free amino-acid composition in the two vineyards.

3.2 Metabolomic analysis of berries

To observe any possible change in berries composition related to 501-treatment, metabolomic UPLC-QTOF-MS analysis was performed.

PCA analysis was executed and no clear grouping was evidenced for both the vineyards (Additional Fig. 5, 6). Paiele and Roncaie datasets were elaborated separately using the OPLS-DA to detect any variation between the control and 501-treated berries.

Table 4

Free amino-acid contents (mg Kg⁻¹) in berries of Paiete and Roncaie plants. P- Paiete; R- Roncaie; C- control; T- treated with 501; h- harvest.

Compound	m/z	PCh	PTh	RCh	RTh
Alanine	90	30.8 ± 1.5	26.3 ± 1.5	40.6 ± 2.1	39.1 ± 2.6
Cysteine	122	21.7 ± 1.5	32.5 ± 2 [§]	15.0 ± 1.0	35.2 ± 1.0 [§]
Aspartic acid	134	323.2 ± 8.8	390.1 ± 10	310.3 ± 11	315.5 ± 15
Glutamic acid	148	61.5 ± 6.5	72.4 ± 5	50.05 ± 9	56.9 ± 10
Phenyl Alanine	165	45.7 ± 3.7	57.1 ± 5.6 [§]	64.31 ± 3.5	65.1 ± 3.1
Glycine	76	14.7 ± 1.2	13.5 ± 0.4	12.28 ± 2.8	14.5 ± 0.9
Histidine	155	11.3 ± 1.1	11.0 ± 1.5	13.3 ± 1.2	15.5 ± 1.9
Isoleucin	132	25.5 ± 2.0	22.3 ± 1.6	25.6 ± 1.5	19.5 ± 2.3
Leucine	147	12.8 ± 1.1	10.9 ± 1.5	12.8 ± 1	14.8 ± 1.3
Lysine	132	15.3 ± 1.2	16.0 ± 1.5	13.8 ± 2	13.5 ± 1.2
Methionine	150	1.5 ± 0.2	3.3 ± 0.2 [§]	2.6 ± 0.5	2.9 ± 0.5
Asparagine	133	28.6 ± 2.5	28.5 ± 1.4	24.1 ± 1.7	23.7 ± 1.5
Proline	116	266.2 ± 18.3	209.9 ± 8 [§]	247.8 ± 18	236.8 ± 23
Glutamine	147	6.4 ± 1.5	5.8 ± 1.2	5.8 ± 1	7.3 ± 0.5
Arginine	175	38.2 ± 1.8	28.2 ± 1.5 [§]	34.5 ± 1.5	46.8 ± 0.9 [§]
Serine	106	4.2 ± 0.8	3.3 ± 0.5 [§]	4.1 ± 1.7	4.7 ± 1
Threonine	120	15.6 ± 1.2	14.5 ± 1.1	17.2 ± 3.5	15.7 ± 1.5 [§]
Valine	118	30.8 ± 1.5	25.4 ± 1.5	22.7 ± 1.3	26.5 ± 3.5
Tryptophan	205	47.2 ± 1.5	53.6 ± 1.5	32.6 ± 1.1	54.7 ± 4.1 [§]
Tyrosine	182.1	14.5 ± 1.5	16.3 ± 1.5	18.1 ± 1.2	16.4 ± 1.2 [§]
§ indicates p < 0.05 Treated vs control.					

The models allowed to observe grouping ascribable to control and treated samples of Paiete berries, with the control group in the -x part of the plot (Fig. 4). The samples of the control berries of Roncaie plants are mostly dispersed in the -x -y part of the plots (Fig. 5).

The UPLC-MS results showed the same pattern of metabolites for the berries of both the vineyards (Table 5).

In general, the level of phenolic constituents in the berries of treated plant increased. The amount of epigallocatechin significantly augmented in berries of Roncaie plants after 501 applications, and the pigment violaxanthin boosted in treated berries of both vineyards. Two sulphur containing organic compounds resulted increased in treated berries, but their identity was not elucidated.

Table 5

Differential metabolite changes in berries after application of preparation 501 (PT and RT) compared to controls (PC and RT). Metabolites presenting VIP > 11 were selected, putative identification was obtained considering HR-MS spectra as well as fragmentation obtained by MSe approach.

Retention time	Var ID (m/z)	PTh vs PCh	RTh vs RCh	Identification
9.37	524.9291	+6.0%	+5.3%	C ₂₃ H ₁₁ NO ₄ S ₅
10.36	577.8214	+ 44.8%	+ 40.0%	Procyanidin B type
3.58	163.4913	+9.0%	+6.6%	<i>p</i> -coumaric acid*
6.93	191.1633	+ 43.9%	+ 21.2%	Quinic acid*
8.56	601.8307	+146.9% §	+ 189.6% §	violaxanthin
8.31	726.0222	+ 128.3%	+ 74.8%	C ₂₄ H ₂₂ O ₂₄ S
2.22	305.4691	+69.1%	+69.8% §	Epigallocatechin*
9.52	601.8098	+45.3%	-23.7%	luteoxanthin
3.61	290.3107	+20.2%	+ 14.5%	catechin*
(*) When available, reference compounds were used to confirm findings. § indicates statistical difference p < 0.05.				

Discussion

Biodynamic agriculture differs from organic management in the use of specific preparations, applied on crops or soil in very small amounts. These preparations are claimed to stimulate soil nutrient cycle, photosynthesis in plants and optimal evolution of compost, enhancing both soil and crop quality [18]. Research on biodynamic viticulture revealed similarity with the organic system concerning soil characteristics, plant growth and yield, resource utilization and biodiversity [19]. The use of preparations had minor influences on growth and yield and did not affect the final quality of the grape berries [20].

The application of horn-silica, preparation 501, to the aerial part of the plants is one of the guidelines given by Steiner to accomplish the biodynamic farming. This work was aimed to discover evidences on the effects of 501 distributions in *Vitis vinifera cv* Garganega plants.

Samples of leaves and berries were collected from plants grown in two separate vineyards closely located, but differentiated by type of soil, both in structure and in plant nutrient contents. The leaves samples were collected at two times, six hours after the 501-treatment (sampling 1, May 10) and 11 days later (sampling 2, May 21) to elucidate whether 501-treatment could induce immediate change in leaves contents or a long-lasting effect could be detectable 11 days after the application. Also, analysis was performed in berries sampled at harvest time.

To assess the variations induced by the application of 501, we first examined the leaf chlorophyll content, commonly regarded as a marker of the nutritional status of the plants. The lack of difference on the level of pigments observed between the leaves of control and 501-treated plants confirms the findings of a previous study on biodynamic treated grapevine [9].

Metabolomics is successfully applied in plant science [21, 22], and the technical advances and the possibility to acquire large number of raw data, due to high resolution mass spectrometry coupled with liquid chromatography, allow to achieve inclusive phytochemical profile in plants, opening new research opportunities [23–25]. Multivariate statistical analysis approach is a tool to investigate metabolic alteration in complex samples as plant tissues. Principal Component Analysis (PCA) is one of the most used techniques in multivariate analysis, aimed at reducing a dataset to its main components and visualizing similarities. PCA has been used in several studies to distinguish varietal and/or geographical origin of grape juice and wines [26]. Other type of data elaboration, as PLS-DA and its implemented version OPLS-DA, may give the advantage of an easier interpretation of the models. Most “omics” experimental setups aim at the comparison of samples between a control and a case group (e.g. disease or treatment). The goal of such differential analysis is therefore to build up a model able to distinguish the classes of observations and to provide a meaningful interpretation of the observed differences [27].

We studied the metabolite profiles in leaves and berries by UPLC-QTOF-MS analysis using an untargeted metabolomics approach. Unlike the targeted methods, where most of the metabolites in the matrix are ignored, in untargeted metabolomics the aim is to achieve the widest possible metabolic coverage in an unsupervised manner, including unknown compounds. Consequently, the measured metabolites are not pre-defined and method development and validation follow a workflow different from the targeted analysis [28].

The PCA for leaf metabolites of both the vineyards showed clusterization only due to sampling time, suggesting limited variation in the leaves composition caused by the 501-treatment compared to time-induced changes. However, further elaboration by supervised methods allowed to detect changes due to treatment, avoiding any bias related to sampling time and location. The separate elaboration of the Paele and Roncaie plants gives indication on possible common changes due to treatment in the leaves of the plants at both locations. These results are, up to our knowledge, the first demonstration of metabolomic changes related to biodynamic 501 applications on grape leaves.

The metabolites changes in leaves of Paele and Roncaie plants indicated that secondary metabolism was influenced by 501 biodynamic treatment. The pathway of shikimate leading to the formation of

phenylpropanoids, stilbenoids and flavonoids appears to be triggered in leaves of 501-treated Paiele plants, while the boost of caftaric and caffeic acid in Roncaie plants indicates that mostly the phenylpropanoid biosynthesis is influenced in the treated plants. The great increase in caffeic and caftaric acids could be counterbalanced by the drop of the level of resveratrol and epicatechin in Roncaie leaves. It is known that higher plant tolerance to abiotic stresses is related to increased synthesis of polyphenols, such as phenolic acids and flavonoids [29]. Our results can suggest that the application of preparation 501 may trigger the biosynthesis of antioxidants beneficial to enhance stress tolerance in grapevine plants, although the different variations in the leaf metabolite levels recorded in the plants of the two vineyards may be attributable to a site effect.

Recently, the total amino-acid content was found to increase in healthy grape berries during maturation under biodynamic management compared to integrated treatment [20]. In our study, the total amount of free amino-acids in berries did not vary, although 501 induced significant variations in some amino-acids. However, the lack of consistency in changes between the berries of the two vineyards leads to exclude a common effect of 501 on amino-acids biosynthesis. It is known that the amount of individual amino-acids in berries could vary with variety, location, age, cultural practices, and method of analysis [30].

The berries of the 501-treated plants in both the vineyards showed superimposable pattern of metabolic changes, mostly being identified as phenolics and carotenoids. Observed metabolic changes in berries varied from those found in leaf tissues, and this can be related to the metabolic specificity of the two plant organs. Phenolic compounds are important in grapes due to their protective function against environmental stress and fungal infection [31]. In 501-treated plants of both sites the level of berry phenolic constituents raised, but only violaxanthin increased significantly. Carotenoids are well known photo-protectors in plant tissues and may interfere during the ripening process in grape [32]. Berries metabolically respond to the light by augmenting the level of compounds like polyphenols that have direct antioxidant and “sun-screening” abilities [33]. The rise of epigallocatechin, although only in Roncaie berries, and violaxanthin can be seen as a beneficial effect of the biodynamic treatment in terms of enhanced response to oxidative stress. The grape berry polyphenols are extracted during wine making and could influence colour as well as the sensory perception of wine [34].

Previous paper investigated the phenolics composition in grape berry of cv Pignoletto and cv Sangiovese, comparing conventional, organic and biodynamic management [35]. Catechin and epigallocatechin had comparable content and rutin decreased in biodynamic treatment compared to conventional and organic management in Pignoletto berries. Even Parpinello *et al.* (2019) evidenced no significant differences between organic and biodynamic Sangiovese wines, although the latter presented higher concentrations of malvidin-3-glucoside and catechin [10].

Our results revealed that catechin and epigallocatechin increased in berries of Paiele and Roncaie plants (Table 5), suggesting a homogeneous trend under biodynamic treatment, independent from the location. The 501 treatment promoted a general increase of phenolic compounds, except for kaempferol-glucoside, in berries of both the vineyards. These results are in agreement with Reeve *et al.* (2005) who reported

increased polyphenols in wine obtained from grapes under biodynamic management [18]. The increment of polyphenols in berries of biodynamically grown plants can confirm the hypothesis of an upregulation of metabolites assumed to induce stress resistance [18, 19]. Higher levels of flavonoids and anthocyanins, with the consequent higher antioxidant potential, were associated to a lower plant vigour, a feature often reported under biodynamic management [36].

Overall, our data indicate a stimulation of the biosynthetic pathways of phenolics in leaves and berries due to the application of 501.

Conclusions

The present study demonstrates that complementary approaches, combining targeted and untargeted data related to primary metabolism and to secondary metabolism, may offer a new opportunity in the evaluation of the complex and multifactorial effects of biodynamic treatment in viticulture.

The application of 501 to plants of *Vitis vinifera* cv Garganega stimulated modifications on the content of phenolic metabolites in leaves and berries. The level of most of the identified compounds by metabolomic approaches increased in plants treated with biodynamic preparation. A common response to 501-treatment was evidenced in the phenolic constituents of berries, while in leaves the effect was dependent on the site of cultivation. The secondary metabolism contributes to the plant adaptation to the environment. The results obtained in the present study could be considered a starting point to further explore the role of the 501 biodynamic treatment on grapevine secondary metabolism.

Abbreviations

ICP-OES

Inductively Coupled Plasma-Optical Emission Spectroscopy

UPLC-HR-MS

Ultra Performance Liquid Chromatography-High Resolution Mass Spectrometry

Q-TOF

Quadrupole Time-Of-Flight

PCA

Principal Component Analysis

OPLS-DA

Orthogonal Partial Least Square Discriminant Analysis

Chla

Chlorophyll a

Chlb

Chlorophyll b

VIP

Variable Importance on Projection

Declarations

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Not applicable

Authors' contributions

M.M. and S.D. conceived and designed the research. S.S, G.K. and S.D. performed the experiments and analysed the data. M.M, S.S., S.D wrote the manuscript. All authors read and agreed to the final version of the manuscript.

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Availability of data and materials

All datasets presented in the current study are available from the corresponding author on request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Authors' details

¹DAFNAE Department of Agronomy Animal Foods Natural resources and Environment University of Padova - Viale dell'Università, 16, 35020 - Legnaro PD - Italy

²DSF Department of Pharmaceutical and Pharmacological Sciences, University of Padova - via Marzolo 5 35121 - Padova - Italy

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Figures

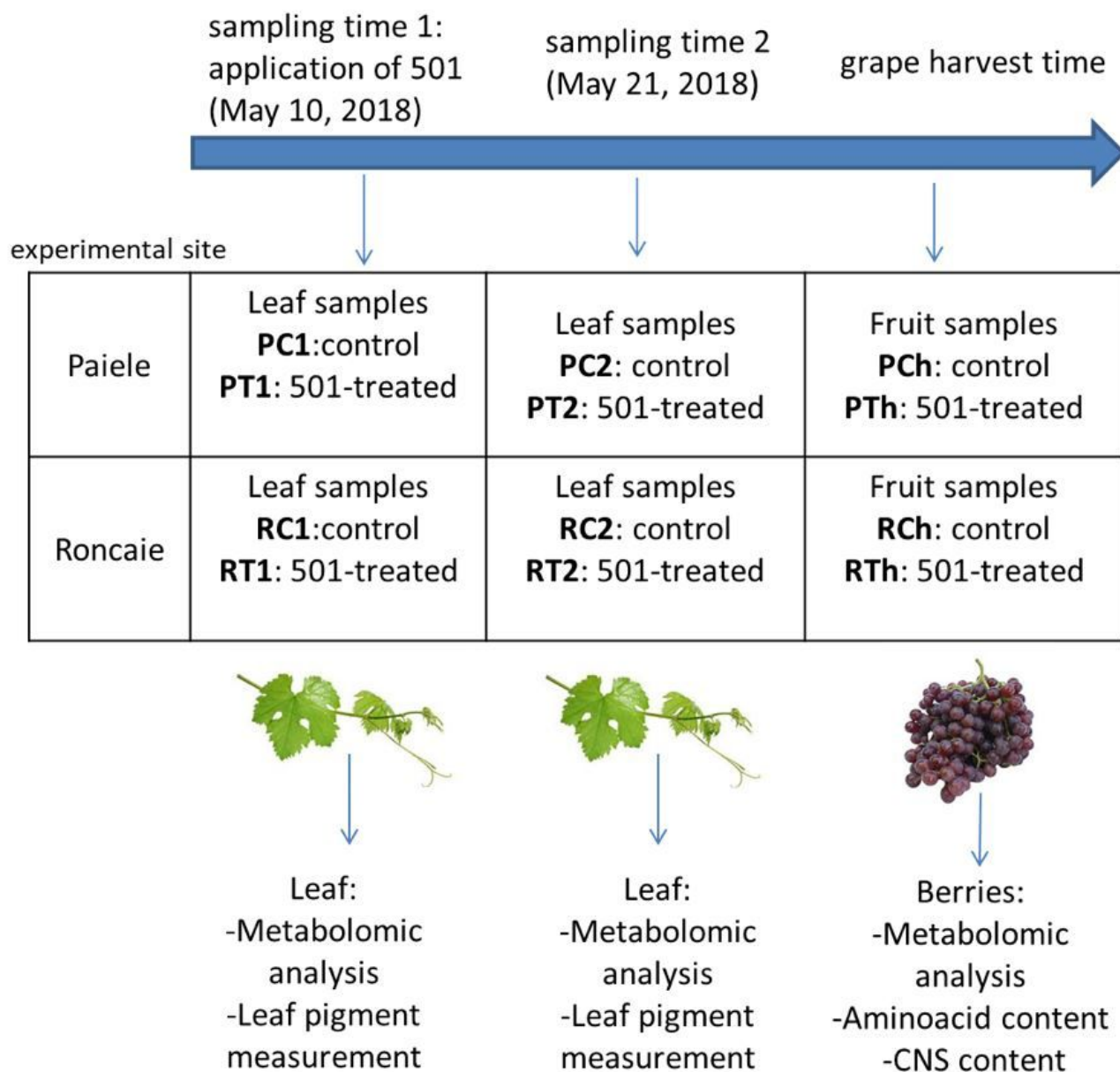


Figure 1

Schematic representation of the sampling time and sample analysis. P- Paiele; R- Roncaie; C- control; T- treated with 501; 1-sampling date May 10; 2-sampling date May 21; h-harvest time.

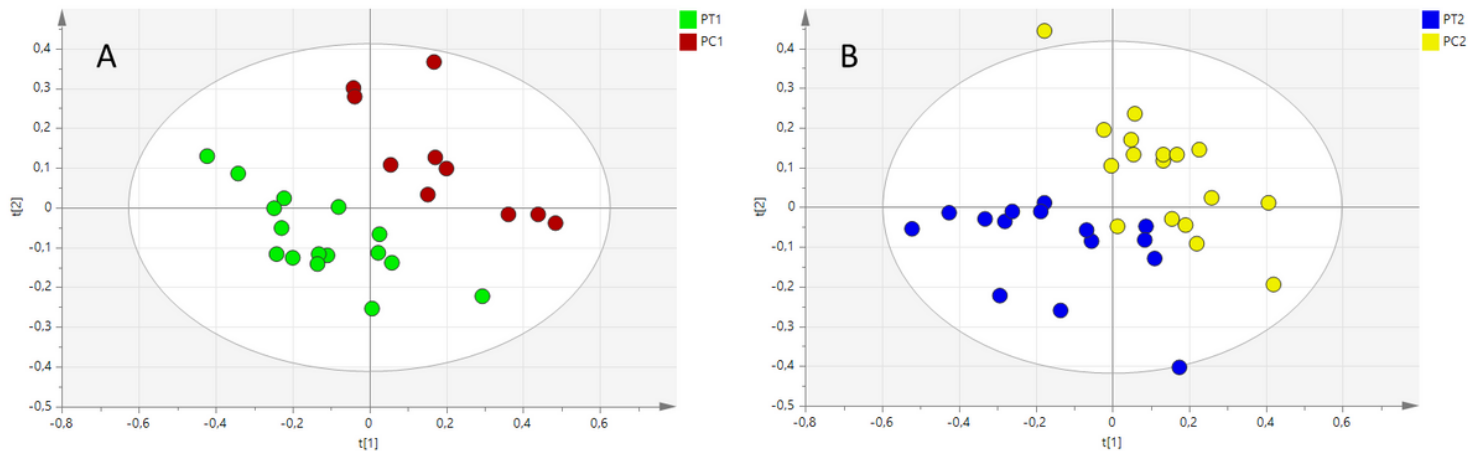


Figure 2

OPLS-DA model for metabolites in leaves of plants in Paele vineyard, comparing control (PC) and 501-treatment (PT) at the sampling time 1 and 2. PC1vsPT1(A) PC2vsPT2 (B). P- Paele; C- control; T- treated with 501; 1- sampling date May 10; 2- sampling date May 21.

Figure 3

OPLS-DA model for leaves of plants in Roncaie vineyard, comparing controls (RC) and 501-treatment R(T) at the sampling time 1 and 2. RC1vsRT1 (A) RC2vsRT2 (B). R- Roncaie; C- control; T- treated with 501; 1- sampling date May 10; 2- sampling date May 21.

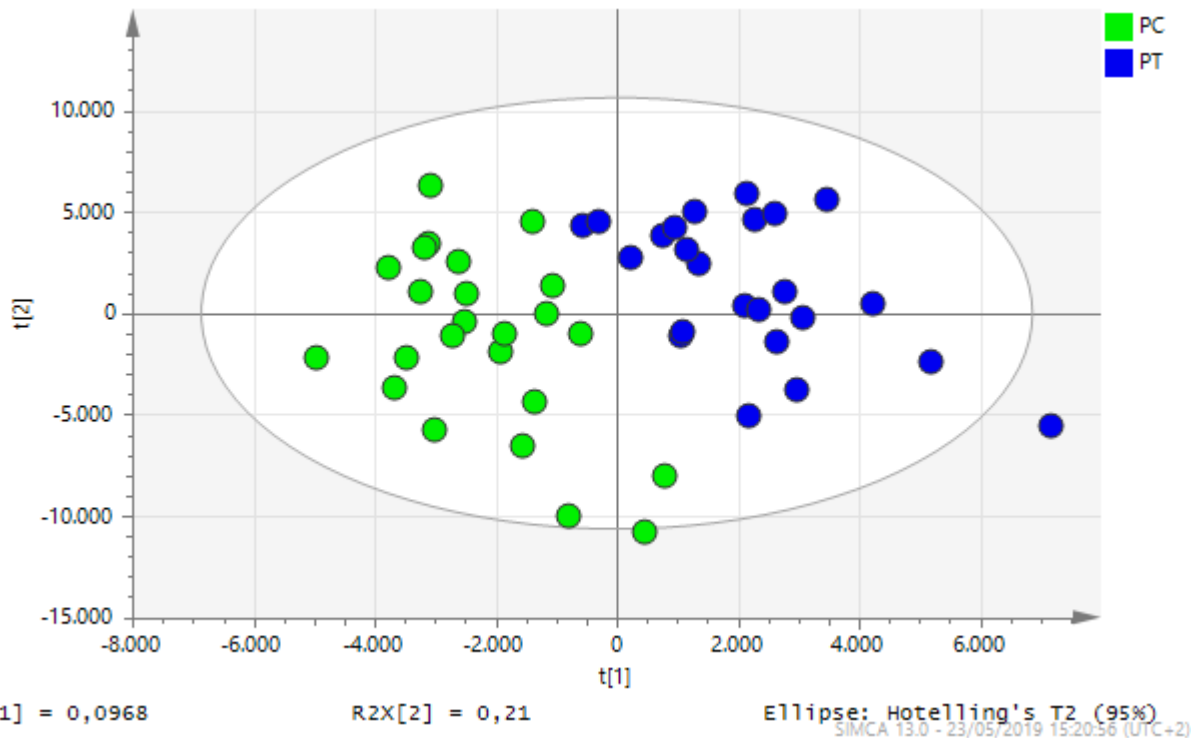


Figure 4

OPLS-DA of berry metabolites in Paele plants. P- Paele; C- control; T- treated with 501.

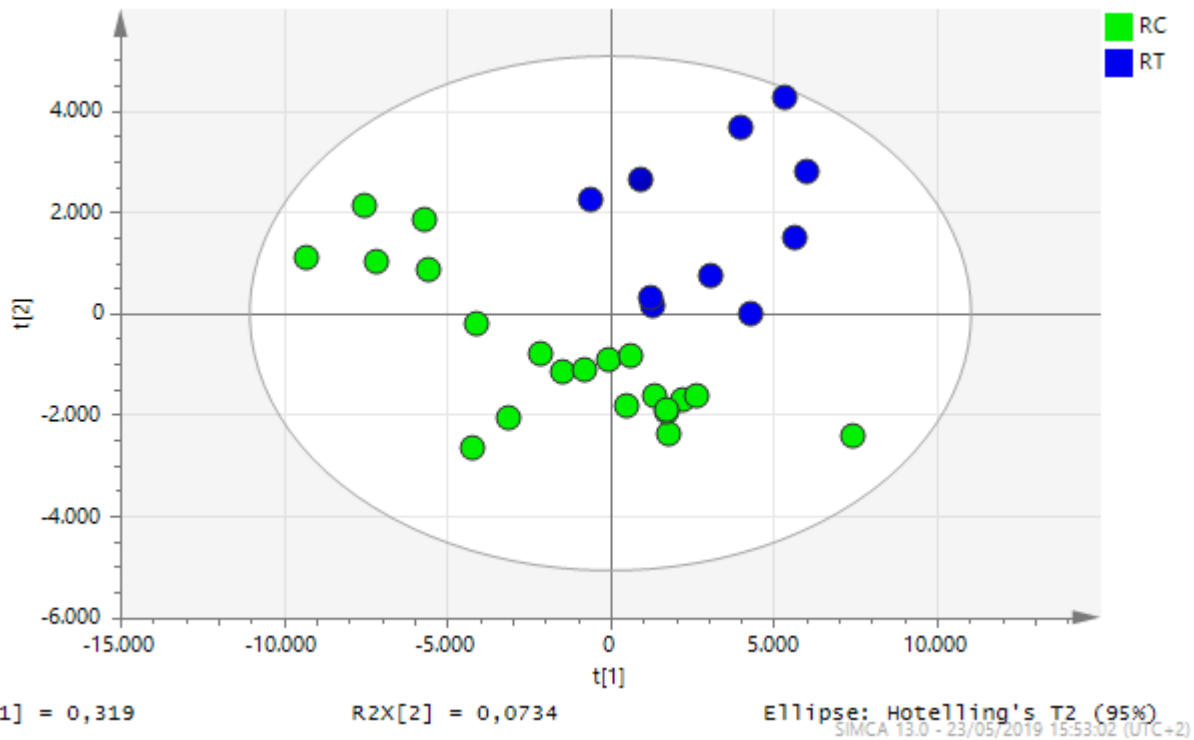


Figure 5

OPLS-DA of berry metabolites in Roncaie samples. R- Roncaie; C- control; T- treated with 501.

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