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Published Version:

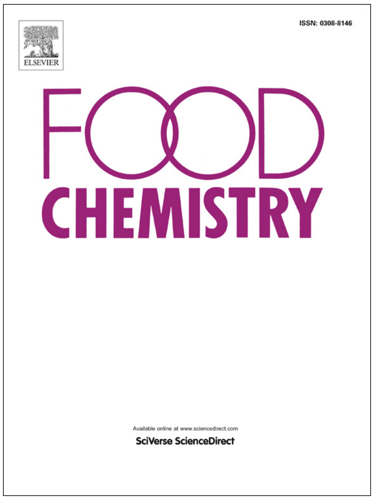
Picone, G., Trimigno, A., Tessarin, P., Donnini, S., Rombolà, A.D., Capozzi, F. (2016). 1H NMR foodomics reveals that the biodynamic and the organic cultivation managements produce different grape berries (Vitis vinifera L. cv. Sangiovese). FOOD CHEMISTRY, 213, 187-195 [10.1016/j.foodchem.2016.06.077].

Availability:

This version is available at: https://hdl.handle.net/11585/548099 since: 2016-07-07

Published:

DOI: http://doi.org/10.1016/j.foodchem.2016.06.077



^{1}H-NMR foodomics reveals that the biodynamic and the organic cultivation managements produce different grape berries (Vitis *vinifera* L. cv. Sangiovese)

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PII: S0308-8146(16)30970-0
DOI: http://dx.doi.org/10.1016/j.foodchem.2016.06.077
Reference: FOCH 19418

To appear in: *Food Chemistry*

Received Date: 19 February 2016
Revised Date: 5 June 2016
Accepted Date: 22 June 2016

Please cite this article as: Picone, G., Trimigno, A., Tessarin, P., Donnini, S., Rombolà, A.D., Capozzi, F., ^{1}H-NMR foodomics reveals that the biodynamic and the organic cultivation managements produce different grape berries (Vitis *vinifera* L. cv. Sangiovese), *Food Chemistry* (2016), doi: http://dx.doi.org/10.1016/j.foodchem.2016.06.077

Title

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Running Title

Foodomics and biodynamic grape berries

Authors

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Highlights

- The influence of biodynamic agronomical practice on grape composition is studied.
- A shift towards a more anaerobic metabolism in biodynamic berries is observed.
- Proline, valine and isoleucine concentrations are higher in biodynamic grapes.

Abstract

The increasing demand for *natural* foods and beverages, i.e. prepared by excluding synthetic chemicals along the whole production chain, has boosted the adoption of organic and biodynamic cultivation methods which are based on protocols avoiding use of synthetic pesticides. This trend is striking in viticulture, since wine production is largely shaped by the varying drinking attitudes of environment-friendly consumers. Using ^{1}H-NMR, the compositions of grape berries, collected at harvest in 2009 and 2011, in experimental plots cultivated either with biodynamic or organic methods, were compared. Although the analysis provides a comprehensive metabolic profile of berries, the resulting distinctive pattern consists of a few molecules. Lower content of sugars, coumaric and caffeic acids, as well as higher amount of γ-aminobutyric acid (GABA) were observed in biodynamic grapes. The ^{1}H-NMR foodomics approach evidenced a diverse fruit metabolome that could be associated to a different physiological response of plants to the agronomic environment.

Keywords

Foodomics; Grape; Organic; Biodynamic, HR-NMR; Metabolomics; GABA, Polyphenols.

1. Introduction

The increasing demand for natural food products (Murdoch & Miele, 1999) has boosted the diffusion of alternative agronomical strategies, such as organic and biodynamic farming (Ponzio, Gangatharan, & Neri, 2013; Willer, Lernoud, & Schlatter, 2014), which can provide numerous benefits in terms of sustainability and soil quality (Gattinger, Muller, Haeni, Skinner, Fliessbach, Buchmann, et al., 2012; Mäder, Fliessbach, Dubois, Gunst, Fried, & Niggli, 2002; John P Reganold, Alan S Palmer, James C Lockhart, & A Neil Macgregor, 1993). This trend is remarkable in viticulture (Loveless, Mueller, Lockshin, & Corsi, 2010; Mann, Ferjani, & Reissig, 2012; Zucca, Smith, & Mitry, 2009), mainly as a consequence of the cultural and social prominent role of wine and of the special attention historically devoted to viticulture and winemaking. Over 280,000 ha of organic grapes were grown in 2014, according to FIBL and IFOAM surveys (Willer, et al., 2014). Worldwide approximately 147,000 ha are managed according to Demeter biodynamic standards (Demeter, 2012). There are 520 Demeter wineries in the world, with 8000 ha of vineyards. Organic and conventional farming adopt distinct management practices and processing methods (John P. Reganold, Alan S. Palmer, James C. Lockhart, & A. Neil Macgregor, 1993), which in many countries are regulated by specific laws. For example, the European Community recently enacted a regulation (EC, 2012) which states that "organic wines" should only originate from organic grapes with limited use of sulphur dioxide during the vinification process and storage, whereas other oenological practices are either restricted or prohibited. By contrast, there is still a lack of official European regulation for biodynamic viticulture and winemaking.

Regardless of certification procedures, biodynamic agriculture differs from organic management in the use of specific fermented preparations, applied on crops or soil in very small amounts, which are claimed to stimulate soil nutrient cycle, photosynthesis and optimal evolution of compost, enhancing both soil and crop quality (Koepf, Pettersson, & Schaumann, 1976; Koepf, Schaumann, & Haccins, 2001).

Few scientific studies have been devoted to understand the effects of biodynamic preparations/method on plant physiology and yield (Botelho, Roberti, Tessarin, Garcia-Mina, & Rombolà, 2015; Collins, Penfold , Johnston, Marschner, & Bastian, 2015; Döring, Frisch, Tittmann, Stoll, & Kauer, 2015; Rombolà, Tassarin, Tumbarello, Parpinello, & Versari, 2015), edible parts composition and nutritional value (Turinek, Grobelnik-Mlakar, Bavec, & Bavec, 2009). Recently it has been reported that some biodynamic preparations led to an increase in leaf enzymatic activities of chitinase and β-1,3-glucanase, which are typically correlated with plant biotic and abiotic stresses and associated with induced plant resistance (Botelho, et al., 2015; Reeve, Carpenter-Boggs, Reganold, York, McGourty, & McCloskey, 2005).

Regarding to yields, both Reeve, et al. (2005) and Botelho, et al. (2015) detected similar values when the organic cultivated vines where compared to the biodynamic ones. In addition, Döring, et al. (2015) observed similar yields in vines under biodynamic and organic farming; however, these cultivation methods showed lower values when compared to the integrated management. Scant information is available on the characteristics of biodynamic grapes and wine (Collins, et al., 2015; Parpinello, Rombolà, Simoni, & Versari, 2015; Plahuta & Raspor, 2007; Reeve, et al., 2005; Ross, Weller, Blue, & Reganold, 2009; Tassoni, Tango, & Ferri, 2013, 2014).

With regard to berry chemistry, a pioneering study from Reeve, et al. (2005) showed that biodynamically cultivated wine grapes had significantly higher soluble solids, total phenols and anthocyanins in the last harvest year compared to those from organically managed plants. In the white cv. Riesling no differences on must soluble solids, pH and total acidity were detected among organic, biodynamic and integrated treatments, whereas α-amino-acids changed according to the cultivation method (Döring et al., 2015).

A study by Tassoni, et al. (2013) compared the biogenic amine content and the polyphenol profiles of berries (cvs. Pignoletto and Sangiovese) grown with conventional, organic and biodynamic practices, highlighting no significant differences among samples. In a recent work with other cultivars (Albana and Lambrusco), Tassoni, et al. (2014) recorded changes in the concentration of catechins and stilbens. However, the analysis did not generally evidence significant differences among wine samples coming from different agricultural and winemaking practices.

A recent study demonstrated that the quality of cv. Sangiovese red wines was largely influenced by the application of biodynamic preparations (Parpinello, et al., 2015). Moreover, by using ^{1}H-NMR, it was possible to discriminate between red wines from organic and biodynamic grapes (Laghi, Versari, Marcolini, & Parpinello, 2014).

The present paper, based on the foodomics approach (Picone, Laghi, Gardini, Lanciotti, Siroli, & Capozzi, 2013; Picone, Mezzetti, Babini, Capocasa, Placucci, & Capozzi, 2011; Savorani, Picone, Badiani, Fagioli, Capozzi, & Engelsen, 2010), proposes a holistic approach combining high resolution nuclear magnetic resonance spectroscopy (HR-NMR) with multivariate data analysis, to reveal differences between the metabolomes of organic and biodynamic grape berry. To date, there are no studies using NMR and foodomics to evaluate the effect of the biodynamic agronomical practices on the grape berry metabolic composition.

NMR spectroscopy is a method of choice for foodomics, due to ease of sample preparation, reducing the risks of manipulation artefacts, and shortening the overall analysis time, largely used in high-throughput applications (Laghi, Picone, & Capozzi, 2014). Despite the lower sensitivity, with respect to some other techniques commonly applied for metabolite profiling, NMR spectroscopy has the advantage to acquire spectra that directly represent the molecular composition of the extracts. NMR spectra, indeed, may be subjected to immediate data analysis, taking advantage of their extreme reproducibility, high dynamic concentration range, and the universal correlation between the concentration and the area of the corresponding signal, as the response factor is the same for every hydrogen atom irrespective of the molecule it belongs to (Laghi, Picone, et al., 2014). Thus, the NMR spectrum of a biological extract reflects, as a whole, its metabolic status, while the loss of important information is reduced as much as possible.

Most of the redundant information associated to the NMR spectra is condensed in a few orthogonal patterns by application of multivariate data analysis. Such a simplification allows the recognition of meaningful information about the response of an organism to external perturbations (Nicholson, Connelly, Lindon, & Holmes, 2002). The main aim of the current study is to measure the overall molecular profile of biodynamic grapes and compare it to the organic ones. The proposed approach applies metabolomics to evaluate the metabolic profile of grapes, as determined on their perchloric acid extracts (Nicholson, et al., 2002). The changes induced by agronomic techniques on the composition of the fruit are exploited in order to interpret the nature of the chemical perturbations. To the best of the authors' knowledge, this is the first study which, starting from the analysis of the overall NMR spectra of samples produced with different agronomic practices, identifies a pool of compounds able to discriminate between grape berries produced by biodynamic and organic cultivation methods. We discuss here possible interpretation of mechanisms of action underlying the observed modifications.

2. Material and methods

2.1. Site description, experimental design and vineyard management

The experiment was performed in a mature vineyard of cv. Sangiovese (clone FEDIT 30 ESAVE), *Vitis vinifera* L., grafted on Kober 5BB, trained to Cordon du Royat. The vineyard was located in Tebano, Italy (44°17′7″ N, 11°52′59″E, 117 m a.s.l.), on a medium hill slope, with South-East/North-West and downhill oriented rows. Vines were spaced in 2.8 m x 1.0 m (3,571 plants ha^{-1}). Starting in 2007, the vineyard was managed as organic (ORG) in accordance with Regulation EC 834/2007 (EC, 2007).

Then, in 2008, the total surface (20,000 m^2) was divided in two large uniform areas with similar soil physic-chemical characteristics, each of them submitted to a specific cultivation method: organic (ORG) farming, managed according to the same Regulation CE 834/2007 (EC, 2007); biodynamic (BD) farming, based on organic management, except for the applications of biodynamic preparations. Each treatment, ORG and BD, was applied to 7 replicates, for a total of 14 experimental plots, each including 12 sample plants (total 168 sample plants). The biodynamic preparations 500, fladen, 500 K, 501 were used from 2008 until 2011 and trunk paste in 2010, as described in Botelho, et al. (2015). To prepare fladen, a hole was dug, delimited by sticks of birch, filled with 50 l fresh and compact cow manure, without straw and subsequently inoculated with compost preparations. Eggshells (100 g) and basalt (500 g) powders were mixed together for 1 h with a spade, then placed in the ground, inside a box with its base open to the soil, for 8 weeks. The fladen preparation was used when dark brown, crumbly and free of manure smell.

In 2008, fladen was applied 3 times, twice in spring and once in autumn; 501 4 times during summer; 500 once in autumn. In 2009, fladen was sprayed 3 times in spring; 500 4 times in spring; 501, twice in summer; 500 K twice in autumn. In 2010, fladen was applied twice in spring; 500 twice in spring, 501 three times in summer, 500 K once in autumn. In 2011 500 was applied twice in spring; fladen twice in spring; 501 three times in summer; of 500 K once in autumn. The application time was modulated according to environmental conditions and observations on plants. In 2008–2011, the number of buds (12–14) was adjusted by winter pruning, whereas the number of bunches (11–15) was adjusted by cluster thinning only in 2011.

At the end of each vegetative season herbaceous species were sown in alternate planting rows, such as fava bean (*Vicia faba*), barley (*Hordeum vulgare*), subterranean clover (*Trifolium subterraneum*) and mustard green (*Brassica juncea*) in both ORG and BD plots. Soil was managed by mowing the vegetation during late spring, which maintained biomass on the soil surface. Both, ORG and BD experimental plots were treated against diseases and pests in the same manner, using the biological products that are allowed by Regulations EC 2092/91 (EC, 1991). Accordingly, treatments consisted mainly of copper (an average of 6 kg/ha/yr) and sulfur (an average of 70 kg/ha/yr), enabling control of fungal pathogens. In 2009, the average T recorded from the end of April to harvest (September 22nd) was 22.0 °C; maximum T of 38.7 °C occurred on July 22nd, total rainfall was 190 mm and predominantly took place in April and during the first week of July. Overall, the 2011 vegetative season was marked by average temperatures well above the seasonal normal, with temperature peaks of 30°C in August. From bud burst to harvest, the average relative humidity (RH) varied from 40 to 70%; highest values were observed during spring (92%) and the lowest (38%) during the latter part of August. The total rainfall (204 mm) from bud burst to harvest was sporadic during spring and almost absent during ripening.

2.2. Analysis of berries

At harvest, on 50 berries randomly collected in each experimental plot, the following parameters were evaluated: berry weight; expressed as g per berry (Gibertini Elettronica S.r.l., Milan, Italy); total soluble solids (TSS; Brix; Electronic Refractometer Maselli Misure S.P.A., Parma, Italy), titratable acidity (TA; expressed as g/l) and pH (Crison Compact Titrator, Crison Instrument SA, Barcelona, Spain). At harvest, two sub-samples of 50 berries per plot were randomly collected from the same side, North-West oriented, of the vegetative wall. The samples were brought on ice to the laboratory and stored at -80° C until extraction.

2.3. HR ^{1}H-NMR sample preparation

Polar metabolites were extracted from a single berry per time (2009 and 2011) using a 3M perchloric acid (PCA, 65%, Sigma-Aldrich Inc., St. Louis, MO, USA) to stop enzymatic activity and to remove proteins (Kruger, Troncoso-Ponce, & Ratcliffe, 2008; Mulas, Galaffu, Pretti, Nieddu, Mercenaro, Tonelli, et al., 2011). Subsequently, the acid mixtures were subjected to a first centrifugation for 5 min at 14,000 rpm (Microfuge® 18 centrifuge, Beckman Coulter, Brea, California) in order to remove insoluble tissues. Each resulting supernatant was dispensed in 1.00 ml aliquots and neutralized using 9 M KOH to pH 7.5 and then centrifuged at 8000 rpm for 5 min at 4 °C in order to remove potassium perchlorate precipitate. NMR samples were prepared by adding 100 µl of deuterium oxide (D_2O) and 10 µl of 3-TrimethylSilyl-Propanoic-2,2,3,3-d4 acid sodium salt (TSP) at a final concentration of 1.4 mM to 600 µl of supernatant.

2.4. ^{1}H-NMR spectra acquisition and processing

All NMR spectra were recorded at 300 K on a Bruker US+ Avance III spectrometer operating at 600 MHz, equipped with a BBI-z probe and a B-ACS 60 sampler for automation (Bruker BioSpin, Karlsruhe, Germany) according to a protocol already published (Picone, et al., 2011). Each spectrum was acquired using 32 K data points over a 7211.54 Hz spectral width and adding 32 transients and an acquisition time of 2.27 s. A recycle delay of 5 s and a 90° pulse of 11.4 μs were set up. HOD residual signal was suppressed by applying the ZGPR sequence (a standard pulse sequence included in the Bruker library) by irradiating it during the recycle delay at δ equal to 4.703 ppm (parts per million by frequency). Each spectrum was automatically processed adjusting phase and baseline with the command apk0.noe using TopSpin version 3.0 (Bruker BioSpin, Karlsruhe, Germany) and applying a line broadening factor of 0.3 Hz. The chemical shifts were internally referenced to the Trimethylsilyl propanoic acid (TSP, Cambridge Isotope Laboratories, USA) at 0.00 ppm. After the Fourier Transformation (FT) and prior to multivariate analysis, data underwent to a pre statistical improvement, such as normalization by Probabilistic Quotient Normalization (PQN) and peaks' alignment by spectral reduction into 192 bins of 0.04 ppm of length. The first step is aimed at removing possible dilution effects (Dieterle, Ross, Schlotterbeck, & Senn, 2006), while the second step avoids the effect of peaks misalignments among different spectra due to variations in chemical shift of signals belonging to some titratable acids. Both these treatments, normalization and binning, were performed by using homemade algorithms written in the R 3.2.2 program language (http://www.r-project.org/). The spectral peaks were assigned by comparing their chemical shift and multiplicity with the literature (Mulas, et al., 2011) and by using Chenomx software (Chenomx Inc., Edmonton, Canada).

2.5. Statistical analysis of berries data

Comparison of means and analysis of variance between treatments were done by using SAS 6.04 software (SAS INSTITUTE, CARY, NC, USA). Means were compared by the Student-Newman-Keuls test ($P = 0.05$).

2.6. Multivariate statistical analysis of NMR data

Multivariate data analysis of the NMR spectra was carried out by using homemade algorithms written in the R 3.2.2 program language. A mean-centred principal component analysis (PCA) was applied on the PQN and binned data set as an explorative analysis to examine the intrinsic variation in the data set.

3. Results and discussion

3.1. Analysis of berries

The cultivation method did not modify berry growth and technological parameters, regardless of the year. In fact, in 2009, BD and ORG vines displayed similar values of berry weight (ORG: 2.15±0.26 g; BD 1.93±0.29 g), TSS (ORG: 22.5±1.6 Brix; BD 20.9±2.6 Brix), pH (ORG: 3.31±0.13; BD 3.3±0.071) and TA (ORG: 4.03±0,46 g l^{-1}; BD: 4.26±0,77 g l^{-1}). Also in 2011 BD and ORG plants showed similar berry weight (ORG: 2.91±0.24 g; BD 1.65±0.281 g), TSS (ORG: 25.5±0.7 Brix; BD 25.6±1.0 Brix), pH (ORG: 3.38±0.049; BD 3.41±0.0) and TA (ORG: 7.58±0.51 g l^{-1}; BD: 7.64±0.61 g l^{-1}).

3.2. NMR analysis.

A representative one dimensional ^{1}H-NMR spectrum of ORG grape berry, from 2009 harvest, acquired from pulp and skin extracts is represented in Figure 1. Twenty-eight metabolites were identified in the spectrum and their assignments are reported in the picture with their relative chemical shifts.

The grape berry extract is characterized by the presence of a high concentration of sugar compounds which resonances in the ^{1}H-NMR spectrum are dominant. This is reflected by their weight as source of variance among all samples, as shown in Figure 2B which represents the loadings plot resulting from PCA applied to the entire data array, according to standard protocols for metabolomics (Laghi, Picone, et al., 2014).

The PC scores plot in Figure 2A shows a tendency of samples to differentiate along PC1according to the cultivation method (BD and ORG), and along PC2 according to the year (2009 white and black circles and 2011 white and black squares). As sugar compounds are abundant in grape berry, PC1 loadings' plot is mainly characterized by sugar resonances. Positive loadings, in the sugar region, mainly involve glucose and fructose signals. Thus, it appears that sugars are more abundant in ORG than in BD, since the latter are in the negative right part of the plot. This trend is confirmed for both 2009 and 2011 years (Table 1 and 2).

In order to have information from the other parts of the spectrum, a PCA was applied separately on both aliphatic (Figure 3) and aromatic (Figure 4) regions.

The loadings' bar-plot along PC1 highlights the metabolites of the aliphatic spectral region contributing to the differentiation between BD and ORG grape berries. Most of these, especially organic acids malate and lactate, are more abundant in the BD samples. Although the differences between refractometric °Brix index of ORG and BD grapes are not statistically significant, it has to be pointed out that the water content may be the main source of variance, thus enlarging the standard deviations of each group masking possible differences among groups. The NMR data, operated in a way that spectra are normalized on the organic compounds content, indeed point out that a different amount of water among berries (dilution factors) are responsible for a large intra-group variance, preventing the differences between groups to be statistically significant. The PQN algorithm, so far adopted to normalize spectra, emancipates from dilutions. Together with organic acids, also some amino acids such as proline, valine and isoleucine are much higher in biodynamic samples together with γ-aminobutyric acid (GABA) (Figure 3B). As far as the aromatic compounds are concerned, it is worth noting here that only molecules soluble in the water phase are extracted and visible in the NMR spectra. Although the intensity of signals reflects a poor abundance with respect to organic acids and sugars, a multitude of peaks is present revealing a complex mixture of monomeric phenolic compounds as evidenced by the PC analysis confined on the aromatic part of the NMR spectrum with exclusion of the formic acid singlet (Figure 4). Along the main component (PC1), which explains the 45% of the total variance, there is a clear separation of the two groups, BD and ORG. The analysis of the loadings (Figure 4) confirms that ORG samples are characterized by higher levels of coumaric acid (Figure 4C) and trans caffeic acid (Figure 4D).

The experimental conditions (experimental design, number of replicates, sampling methods, etc.), adopted in the present study, allowed us to minimize possible interference exerted by environmental and agronomical factors in the comparison of the cultivation method. The vineyard hosting the trial was accurately controlled by monitoring physiological parameters (e.g. leaf chlorophyll and nutrient concentration, photosynthetic activity, leaf water potential), yield and fruit quality. Plants subjected to both BD and ORG methods displayed similar vegetative (e.g. pruning weight), productive (e.g. yield) (data not shown) and technological parameters (e.g. berry soluble solids, pH and titratable acidity), the latter being of practical use for oenologists, although such parameters do not contain details about molecular composition. Therefore, the differences detected by ^{1}H-NMR on berries from the two cultivation methods should be ascribed to the use of biodynamic preparations.

Based on the results of this work it can be referred that berries from BD agriculture, compared to those from ORG cultivation, display an activation of glycolysis toward fermentative pathway. The fermentative pathway, occurring under the absence or low oxygen concentration, implies that pyruvate is converted into lactate or ethanol. Under non limiting oxygen levels, pyruvate is oxidized to yield acetyl CoA that enters in the Krebs cycle. Indeed, the lower concentration of sugars (mainly glucose and fructose) along with the increase concentration of organic acids such as lactate and malate and ethanol might suggest the activation of the anaerobic metabolism (Vigani, Chittó, De Nisi, & Zocchi, 2012) in the BD berries respect to ORG berries. Grapevines of cv. Sangiovese, under BD management exhibited lower leaf stomatal conductance as compared to organically cultivated vines (Botelho, et al., 2015). In a study conducted on cv. Riesling this parameter did not differ between ORG and BD vines (Döring et al., 2015). The different effects of the cultivation method could be related to plant water status and genotypic responsiveness toward it (Chaves et al., 2010). Furthermore, reduction in stomatal conductance has been associated with enhanced plant tolerance toward biotic (Zeng, Melotto, & He, 2010) and abiotic stresses (Salazar-Parra, Aguirreolea, Sánchez-Díaz, Irigoyen, & Morales, 2012), which is considered to be one of the key aspects of BD viticulture (Turinek, et al., 2009). A lower stomatal conductance is associated with an increase in the concentration of internal CO_2. These findings might support the possible predominance of anaerobic metabolism in BD berries. Accordingly, the observed accumulation of formic acid in BD berries would result from the activation of formate dehydrogenase that under specific conditions might consume CO_2 and NADH to produce formate. Considering that this reaction might regenerate NAD^+, the accumulation of formate in BD berries might suggests the occurrence of an enhanced turnover of reducing power presumably associated to compensate the deficit of aerobic metabolism (mitochondrial respiration and Krebs cycle) (Vigani & Zocchi, 2010). In fact, together with organic acids, also some amino acids such as proline, valine and isoleucine are much higher in biodynamic samples together with γ-aminobutyric acid (GABA) (Figure 3). In particular, isoleucine and valine are formed from pyruvate: the increase in their concentration in the BD berries suggests that a reduced amount of pyruvate enters in the Krebs cycle and could be directed to other routes. Since the vines submitted to BD cultivation method exhibited lower leaf stomatal conductance (Botelho, et al., 2015), it would be interesting to further explore the possible relationship between this physiological parameter and the fruit anaerobic metabolism.

The ^{1}H-NMR technique, by emancipating from possible detrimental artefacts introduced by more manipulating methods, provided complex information on the chemical composition of berries, embedded in hundreds of signals generated by many compounds. However, the complexity of information is simplified by multivariate data analysis which mines the relevant spectral features responsible for variance, and this approach highlighted the prevalent role of few molecules that clearly discriminated between the two cultivation methods. Among these substances there are some polyphenols, such as coumaric and caffeic acids that represent a class of phenolic acids largely important for their influence on the wine sensory value. In fact, they contributes to the colour, astringency, bitterness and the aroma of wine, either directly or as a result of a whole interactions with other molecules such as proteins, polysaccharides or other polyphenols (Preys, Mazerolles, Courcoux, Samson, Fischer, Hanafi, et al., 2006). In our experiment, coumaric and caffeic acids, together with other polyphenolic compounds, were lower in BD grapes with respect to ORG ones. Thus, between BD and ORG berries there are differences in the polyphenolic profile that may reflect in the deriving wine. Wines derived from BD grapes harvest in 2009 were characterized by a lower concentration of total polyphenols and anthocyanins than ORG wines of the same year (Parpinello, et al., 2015), and the observed trend is confirmed in the corresponding berries in the present work. In spite of their importance in grape and wine quality, polyphenols are hard to compare among different cultivation methods, because their content is heavily affected by environmental and other agronomical practices (e.g. canopy and soil management) as well as vegetal material (e.g. variety, rootstock) (Downey, Dokoozlian, & Krstic, 2006). Therefore, limited published literature is present on this subject. The conclusions drawn by Tassoni, et al. (2013) and (2014) are based on a single investigated vineyard per each cultivation method. Moreover, the concentration of specific polyphenols was not reported by Reeve, et al. (2005).

It is noteworthy that the concentration of GABA, significantly higher in berries obtained by the BD management in the 2009 harvest, effectively discriminated between the two cultivation methods. Although the difference in not significant in 2011, the same trend is observed confirming the tendency to be more accumulated in BD than in ORG. However, it has to be argued that the environmental conditions, fluctuating year by year, may have influence in attenuating such a difference. This molecule appears ubiquitously in living organisms and is considered an indicator of the capability of plants to react to environmental stimuli, including attacks by insects and pathogens (Bown, Hall, & MacGregor, 2002; Ramputh & Bown, 1996; Wallace, Secor, & Schrader, 1984). GABA has different functions within the plant: defense against insects (Bown, et al., 2002; Ramputh, et al., 1996), protection from oxidative stress (Bouché, Lacombe, & Fromm, 2003) and transmission of signals in the plant (Bouché, et al., 2003; Kinnersley & Turano, 2000). In particular, in response to a signal mediated by cytosolic Ca^{2+} ions, GABA is rapidly synthesized in high amounts (Shelp, Bown, & McLean, 1999). In addition, GABA represents a substrate for yeasts. *Saccharomyces cerevisiae* can in fact efficiently use it as a source of assimilable nitrogen, especially in conditions of poor availability of YAN (Bach, Sauvage, Dequin, & Camarasa, 2009). The biodynamic cultivation method induced in wines (Laghi, Versari, et al., 2014) effects the GABA concentration similar to those found in the present paper. The new information may suggest that the source of such a difference could be assigned prevalently to the cultivation practice rather than to yeasts co-metabolism. Consequently, GABA represents a candidate molecule as a possible marker for sustainable agricultural systems. Furthermore, in humans GABA works an inhibitory neurotransmitter with beneficial health effects; hence special attention is devoted to improve its concentration in food and beverages (Abdou, Higashiguchi, Horie, Kim, Hatta, & Yokogoshi, 2006). As GABA is a component of food imparting health benefits (relief from stress, better immune system), biodynamic applications could be of interest, in the future, in the research area pertaining the healthy diet. To our knowledge, this is the first study that highlights changes of GABA in grape berries associated to BD method.

4. Conclusions

The comparison of grape composition between two different cultivation methods demonstrated adaptive changes, involving molecules that may add value to the quality of food, here assigned to specific compounds testifying the capability of plants to respond to challenging surrounding environment. The foodomics approach, previously applied to grape berries to evaluate the link between genotype and metabotype, (Picone, et al., 2011) has been exploited in the present research work to explore the extent of the metabolic perturbation induced by the agronomic practice, when the pedoclimate is kept constant. Previous studies evidenced differences in the metabolic content of wines produced by either BD or ORG cultivations, although they did not resolve whether the differences were already present in the grapes or appearing after winemaking. The present foodomics study, based on 1H-NMR spectroscopic data, which are explored by unsupervised multivariate analysis, confirms that such differences are statistically meaningful in the grape metabolome. Thus, foodomics is shown once again to provide robust information about changes in the molecular composition of food extracts when different production systems are compared.

Acknowledgements

This work was supported by CL.A.N (Cluster Agrifood Nazionale D.D. MIUR 30.5.2012 prot.N.257/RIC) through the PROSIT Project. The Consortium CIRMMP is acknowledged as well for providing support and facilities.

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Figure Captions

Fig. 1. ^{1}H-NMR spectrum registered on a grape berry. The spectrum has been split into three parts of clarity. Some resonances have been assigned and labelled accordingly in the following regions: A) Aliphatic region, characterized by the presence of signals belonging to amino acids and organic acids; B) Carbohydrate region, characterized by the presence of signals belonging to sugars and C) Aromatic region, characterized by the presence of signals belonging to phenolic and other aromatic compounds.

Fig. 2. Multivariate analysis on biodynamic and organic grape berry samples. A) PC score plot from 84 NMR spectra of skins and pulp extract of BD (open symbols) and ORG (filled symbols) berries harvested in 2009 (circle) and 2011 (square). The first two PCs explain 87% (PC1) and 10% (PC2), respectively, of the total variance. B) Loading bar-plot for spectral bins along PC1. C) Boxplot of sugar regions from 3.21 to 4.20 ppm, characterized by the presence of fructose and glucose signals.

Fig. 3. Biodynamic and organic grape berry samples multivariate analysis. A) PC score plot of aliphatic region (from 0.51 to 3.21 ppm) from 84 spectra of extracts from skins and pulp of BD (open symbols) and ORG (filled symbols) berries harvested in 2009 (circle) and 2011 (square). The first two PCs explain 56% (PC1) and 17% (PC2), respectively, of the total variance. B) Loadings bar-plot for spectral bins along PC1. C) Boxplot of GABA interval from 1.82 to 1.93 ppm.

Fig. 4. Biodynamic and organic berry samples multivariate analysis. A) PC score plot of aromatic region (from 5.55 to 8.4 ppm) from 84 spectra from extracts of skins and pulp of BD (open symbols) and ORG (filled symbols) berries harvested in 2009 (circle) and 2011 (square). The first two PCs explain 45% (PC1) and 18% (PC2), respectively, of the total variance. B) Loadings bar-plot for spectral bins along PC1. C) Boxplot of Coumaric Acid interval from 6.87 to 6.99 ppm. D) Boxplot of Trans Caffeic Acid interval from 7.57 to 7.68 ppm.

Table 1. Signal areas (arbitrary units proportional to the concentration) of sugars, coumaric acid, caffeic acid and GABA in cv. Sangiovese berries obtained in 2009 from experimental plots under organic (ORG) and biodynamic (BD) management.

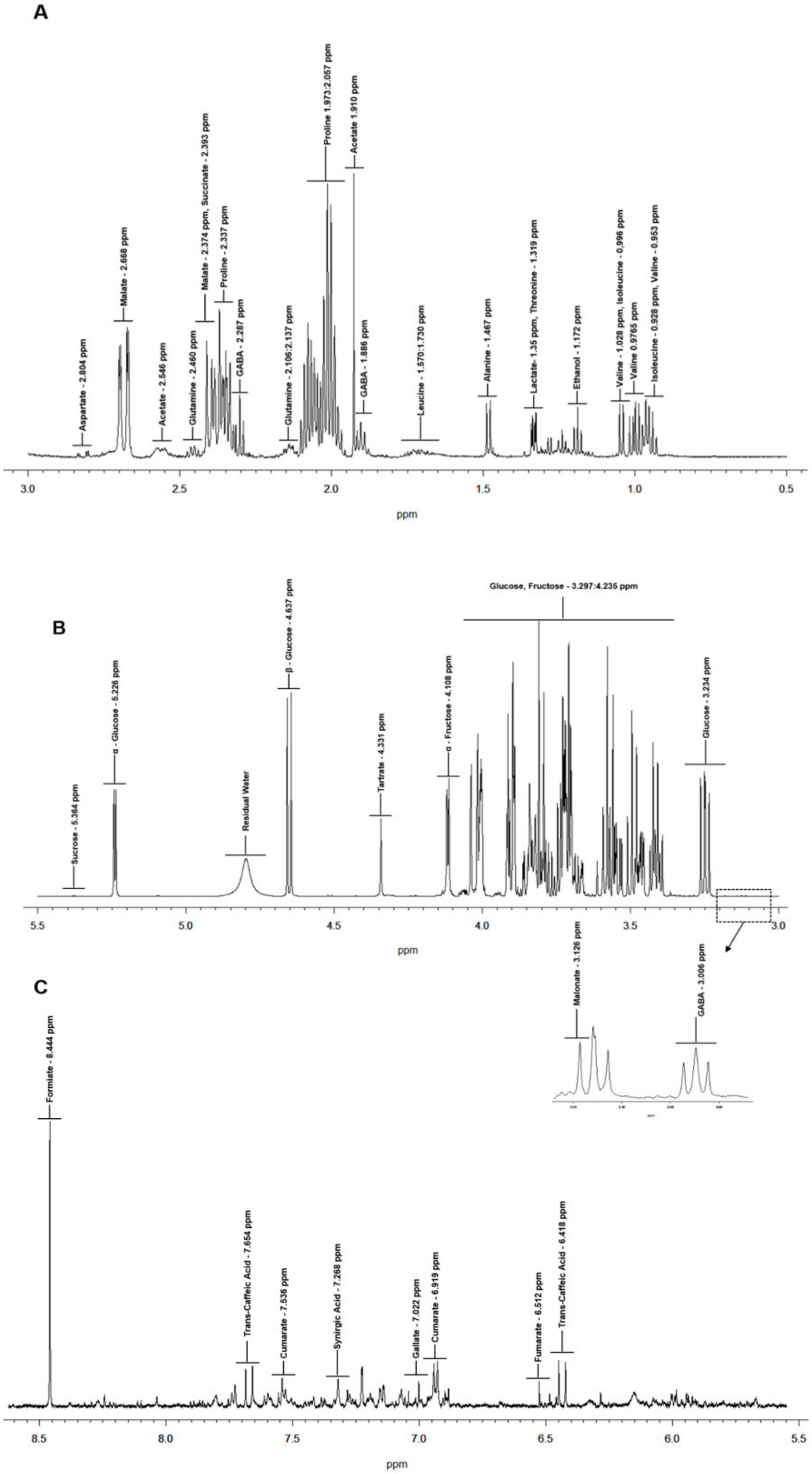
2009				
Treatment	**Sugars**	**Coumaric**	**Caffeic**	**GABA**
ORG	103.501,86	12,696	5,0791	14,478
BD	92.253,39	7,4713	3,2057	18,007
Significance	**	**	***	**

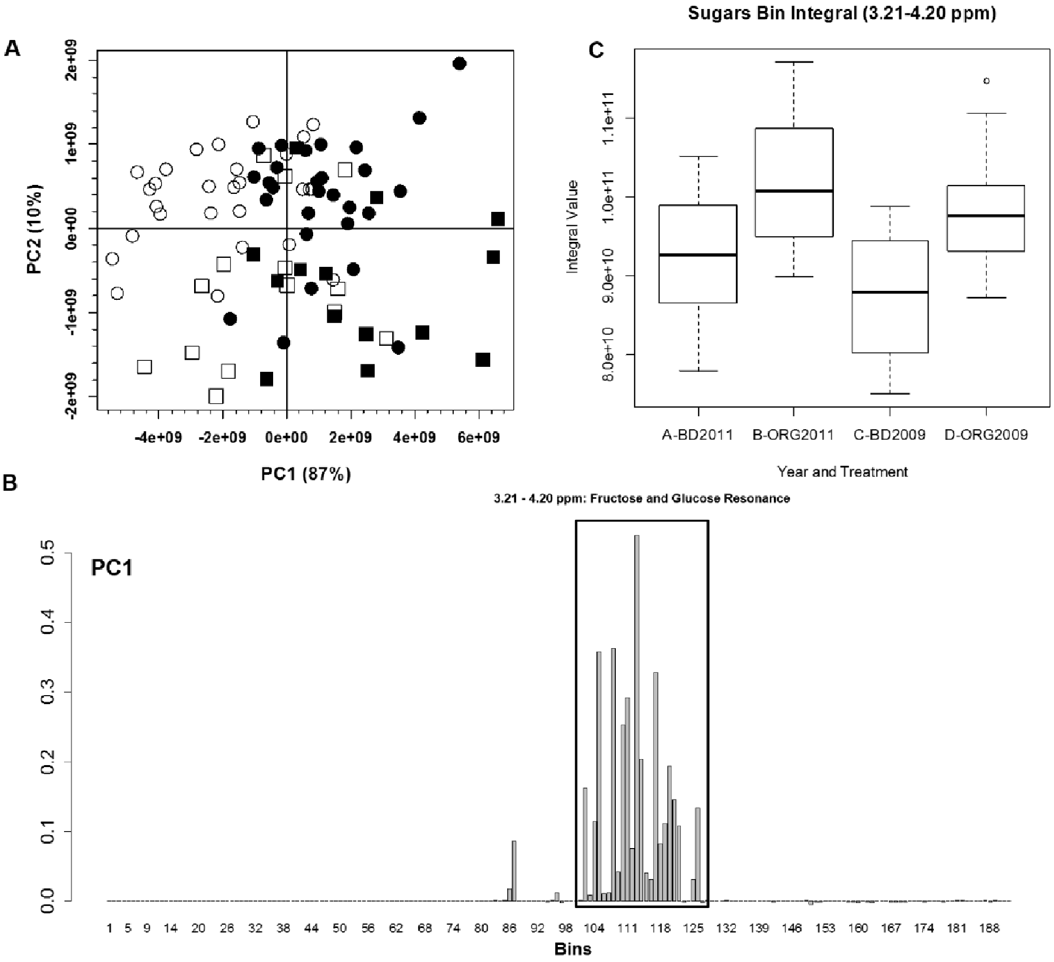
Significant at $P \leq 0.01$; * significant at $P \leq 0.001$.

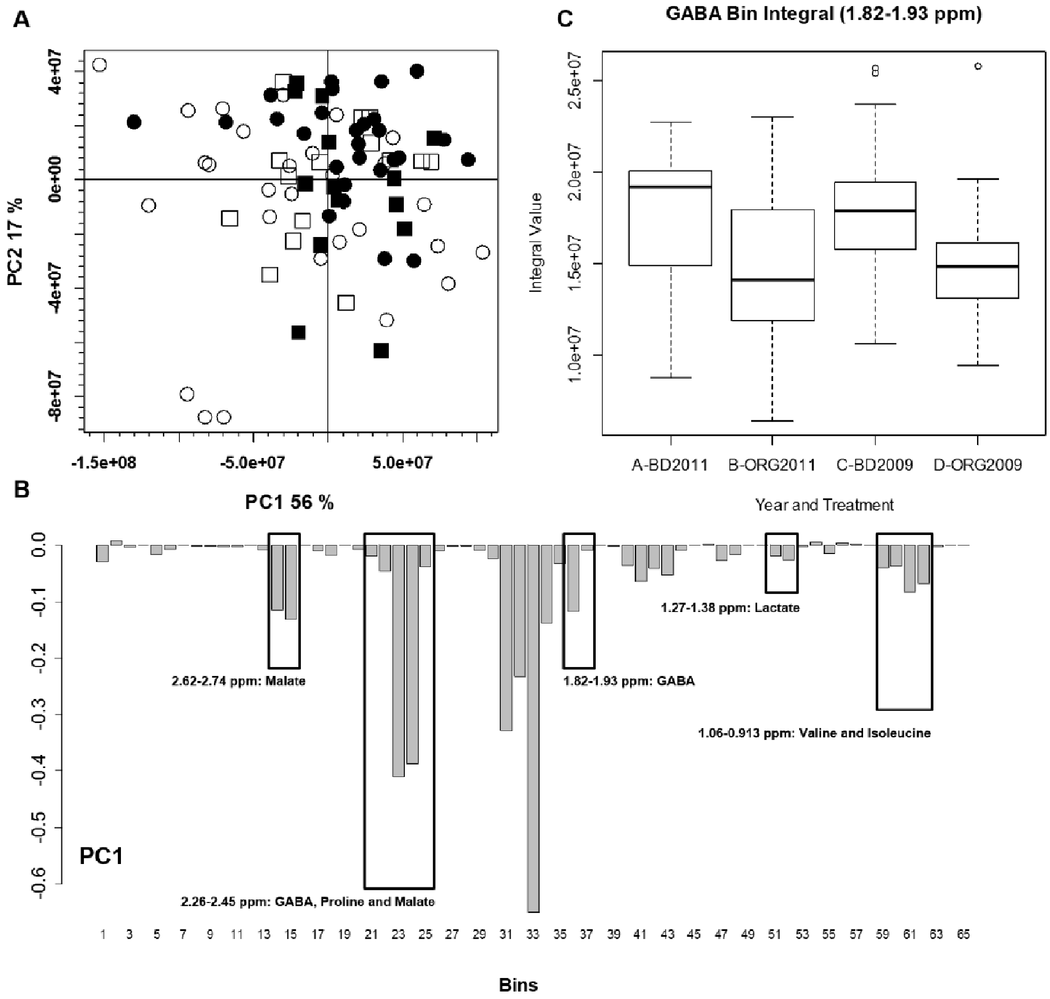
Table 2. Signal areas (arbitrary units proportional to the concentration) of sugars, coumaric acid, caffeic acid and GABA in cv. Sangiovese berries obtained in 2011 from experimental plots under organic (ORG) and biodynamic (BD) management.

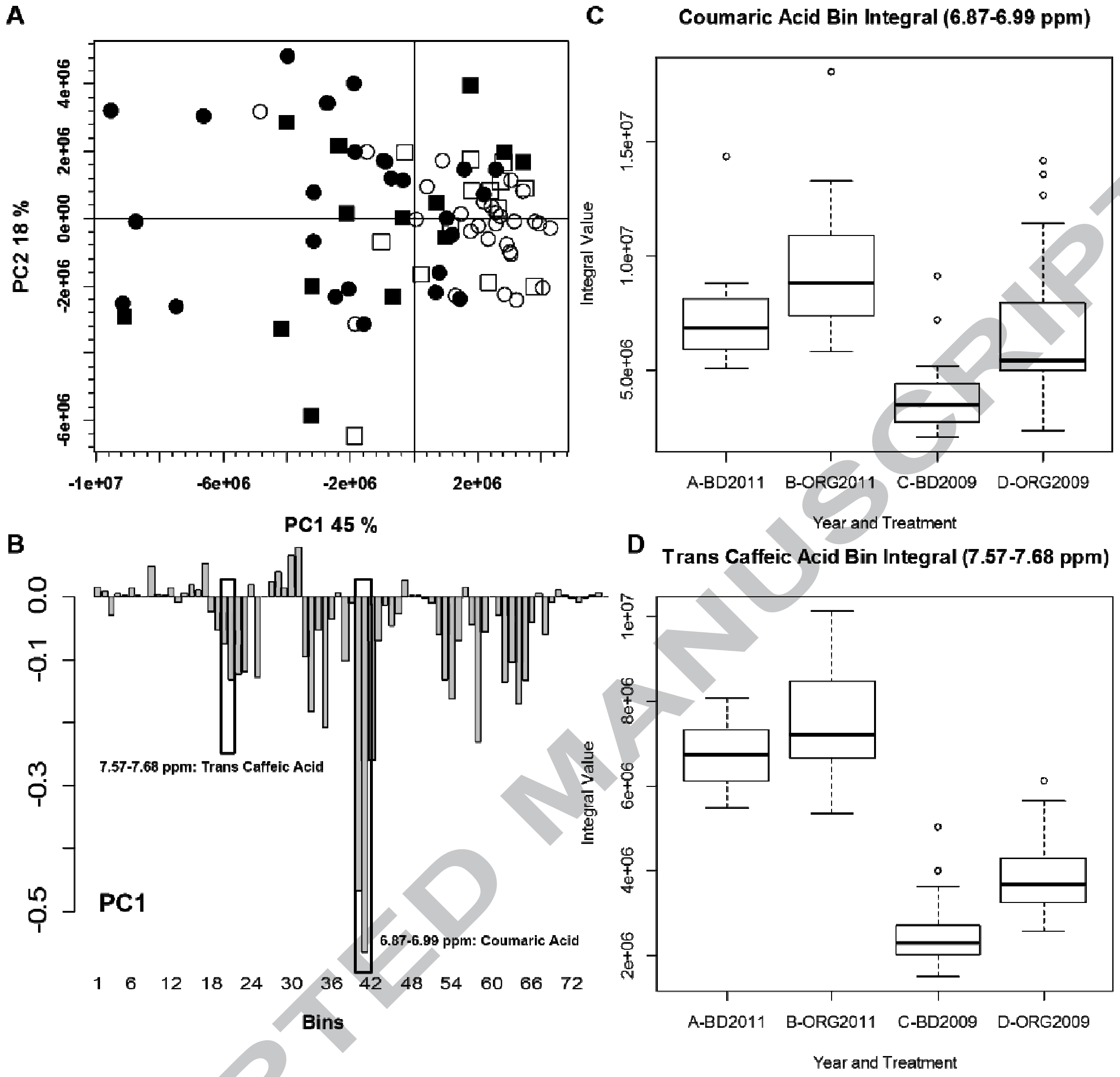
2011				
Treatment	**Sugars**	**Coumaric**	**Caffeic**	**GABA**
ORG	106975,6726	17,656448	9,096597	14,536449
BD	96410,60039	15,207398	8,162619	16,682954
Significance	**	**	**	*n.s.*

** significant at $P \leq 0.01$; n.s., not significant.


A
Aspartate - 2.804 ppm
Malate - 2.668 ppm
Acetate - 2.546 ppm
Glutamine - 2.460 ppm
Malate - 2.374 ppm, Succinate - 2.393 ppm
Proline - 2.337 ppm
GABA - 2.287 ppm
Glutamine - 2.106:2.137 ppm
Proline 1.973:2.057 ppm
Acetate 1.910 ppm
GABA - 1.886 ppm
Leucine - 1.570:1.730 ppm
Alanine - 1.467 ppm
Lactate- 1.35 ppm, Threonine - 1.319 ppm
Ethanol - 1.172 ppm
Valine - 1.028 ppm, Isoleucine - 0.996 ppm
Valine 0.9765 ppm
Isoleucine - 0.928 ppm, Valine - 0.953 ppm
3.0
2.5
2.0
1.5
1.0
0.5
ppm
B
Sucrose - 5.364 ppm
α - Glucose - 5.226 ppm
Residual Water
β - Glucose - 4.637 ppm
Tartrate - 4.331 ppm
α - Fructose - 4.108 ppm
Glucose, Fructose - 3.297:4.235 ppm
Glucose - 3.234 ppm
5.5
5.0
4.5
4.0
3.5
3.0
ppm
Malonate - 3.126 ppm
GABA - 3.006 ppm
C
Formiate - 8.444 ppm
Trans-Caffeic Acid - 7.654 ppm
Cumarate - 7.536 ppm
Synirgic Acid - 7.268 ppm
Gallate - 7.022 ppm
Cumarate - 6.919 ppm
Fumarate - 6.512 ppm
Trans-Caffeic Acid - 6.418 ppm
8.5
8.0
7.5
7.0
6.5
6.0
5.5
ppm


A
PC2 (10%)
2e+09
1e+09
0e+00
-1e+09
-2e+09
-4e+09
-2e+09
0e+00
2e+09
4e+09
6e+09
PC1 (87%)
C
Sugars Bin Integral (3.21-4.20 ppm)
Integral Value
1.1e+11
1.0e+11
9.0e+10
8.0e+10
A-BD2011
B-ORG2011
C-BD2009
D-ORG2009
Year and Treatment
B
3.21 - 4.20 ppm: Fructose and Glucose Resonance
PC1
0.5
0.4
0.3
0.2
0.1
0.0
1 5 9 14 20 26 32 38 44 50 56 62 68 74 80 86 92 98 104 111 118 125 132 139 146 153 160 167 174 181 188
Bins


A
PC2 17 %
4e+07
0e+00
-4e+07
-8e+07
-1.5e+08
-5.0e+07
5.0e+07
PC1 56 %
C
GABA Bin Integral (1.82-1.93 ppm)
Integral Value
2.5e+07
2.0e+07
1.5e+07
1.0e+07
A-BD2011
B-ORG2011
C-BD2009
D-ORG2009
Year and Treatment
B
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
PC1
2.62-2.74 ppm: Malate
1.82-1.93 ppm: GABA
1.27-1.38 ppm: Lactate
1.06-0.913 ppm: Valine and Isoleucine
2.26-2.45 ppm: GABA, Proline and Malate
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61 63 65
Bins


A
PC2 18 %
4e+06
2e+06
0e+00
-2e+06
-6e+06
-1e+07
-6e+06
-2e+06
2e+06
PC1 45 %
B
0.0
-0.1
-0.3
-0.5
7.57-7.68 ppm: Trans Caffeic Acid
PC1
6.87-6.99 ppm: Coumaric Acid
1 6 12 18 24 30 36 42 48 54 60 66 72
Bins
C
Coumaric Acid Bin Integral (6.87-6.99 ppm)
Integral Value
1.5e+07
1.0e+07
5.0e+06
A-BD2011
B-ORG2011
C-BD2009
D-ORG2009
Year and Treatment
D
Trans Caffeic Acid Bin Integral (7.57-7.68 ppm)
Integral Value
1e+07
8e+06
6e+06
4e+06
2e+06
A-BD2011
B-ORG2011
C-BD2009
D-ORG2009
Year and Treatment