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ORIGINAL PAPER

Polyphenols and aromatic volatile compounds in biodynamic and conventional 'Golden Delicious' apples (*Malus domestica* Bork.)

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Abstract Biodynamic and conventional apples of the cultivar 'Golden Delicious' were characterized based on the investigation of polyphenol content and volatile composition. Polyphenols were determined by high-performance liquid chromatography with diode-array detection and mass spectrometry HPLC/DAD/MS analysis; volatile organic compounds (VOCs) were detected with a proton transfer-time of flight-mass spectrometer (PTR-ToF-MS) approach. Colour and physiochemical fruit parameters were also acquired to compare fruit ripeness. By the analysis of the entire data set, it emerges that polyphenols can separate samples both on geographic and agricultural management basis, according to PCA analysis. On the contrary, PCA on volatile compounds is unable to separate the samples. Results suggest that, in apple fruits, polyphenols content is highly influenced by external factors, while volatile profile is under a stronger genetic control, thus more stable across different environments.

Keywords Farming system · HPLC/DAD/MS · *Malus domestica* Bork. · Secondary metabolites · PTR-ToF-MS · VOCs

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Introduction

The influence of the agronomic management on the health-promoting properties of food is at the centre of a great debate; while it is generally accepted that organic agriculture helps in preserving soil fertility with reduced tillage and use of pesticides, there is no general evidence to support the consumer belief that the consumption of organic food enhances his health; however, people consuming organic food show a consistent reduction of exposure to pesticides residues [1, 2]. Furthermore, recently, a meta-analyses based on 343 peer-reviewed publications, indicated statistically significant and meaningful differences in composition between organic and non-organic crops/crop-based foods [2]. In this debate, a particular role is played by biodynamic agriculture. With respect to organic management, biodynamic agriculture is based on a holistic approach of the whole farm and on the use of specific preparations for the soil (preparation 500), plants (preparation 501), and compost (preparations 502–507).

In a relatively moderate number of investigations on metabolic compounds in food from biodynamic agriculture as compared to food from conventional or organic management, the attention has been frequently drawn to certain phytochemicals, such as carotenoids, flavonoids, polyphenols, and anthocyanins [3–6]. Apple fruits (*Malus domestica* Bork.) are regarded as a very rich source of phytochemicals, which may play a role in reducing chronic disease risk [7], and recently, a survey on the phytochemical content of 247 wild and domesticated apple accessions was performed [8]. The polyphenol content of apple has been largely analyzed to determine if agriculture management may affect the qualitative and quantitative composition [2, 9–11]; however, biodynamic management has never been taken into account. Similar consideration can be drawn

concerning aroma compounds (or volatile organic compounds, VOCs); in fact, they have been used as markers of different agriculture management techniques [12], and their crucial role in driving the consumer preferences of food has been well highlighted in the case of apples [13]. Nevertheless, to date, no information can be achieved on how (or if) their quantity (polyphenols) and quality (VOCs) maybe be enhanced by a biodynamic farming approach.

Therefore, fruit characteristics, level of phenolic compounds, and volatile composition were determined in apple fruits (cv. 'Golden Delicious') in the conventional and biodynamic agriculture performed in northern Italy (Trentino Alto Adige) and compared to the conventional apples grown in central Italy (Tuscany), to point out also the role of different pedoclimatic conditions. Polyphenols content was determined with HPLC/DAD/MS, and the volatiles' composition with PTR-ToF-MS, a non-invasive technique which allows the achievement of whole mass spectra with a time of resolution lesser than 1 s and the detection of high molecular mass molecules with a high resolution power ($m/\Delta m \sim 4000$); it also provides unambiguous determination of chemical formula leading to a better interpretation of mass spectra [for an in deep description of PTR-ToF-MS advantages and limits, see 14]. This technique has already been used for apple VOCs analysis as a method for surveying an apple germplasm collection [15], to follow VOCs emission during the apple ripening process [16] and in the study of superficial scald post-harvest disorder [17].

Materials and methods

Plant material and fruit collection

Analyses were performed on ripe apple fruits (Malus domestica, Bork.) harvested from trees of 'Golden Delicious' clone B grafted on M9 rootstocks grown in three commercial orchards, two located in the north of Italy (Trentino Alto Adige, marked as "A" in the following text), and one in central Italy (Tuscany, marked as "B" in the following text). The latter was chosen to verify the pedoclimatic effect on the fruits characteristics. A minimum of 40 fruits, with similar size and without visible external damage, were hand-picked from at least 15 healthy trees for each orchards; the collection was performed during the second week of September 2014, at a commercial ripening stage based on colour change, fruit firmness, total sugar content, and starch index; samples, stored at 4 °C and 80% of relative humidity (RH), were transferred to room temperature (20 ± 3 °C) for 24 h before performing the analysis in the following week. The conventional agronomic management was applied in one orchard in Trentino Alto Adige (CON-A) and in Tuscany (CON-B), while the third orchard (BIO-A) was managed following biodynamic protocols. The Tuscan conventional orchard (2857 plants/ha; 40 t/ha fruit yield) was planted in 2007 on a clay loam soil with a SE-NW field exposure. The two orchards from Trentino Alto Adige were both established in 1998 on adjacent areas, thus under very similar soil (rocky soil texture) and environmental conditions. Planting density is 2032 and 3333 trees/ha, with an average yield of 55 and 85 t/ha of apples, for the biodynamic and conventional orchard, respectively. The biodynamic orchard is run according to the indications of Rudolf Steiner [18], and it is protected from external abiotic and biotic contaminations by living hedges. The soil fertility is maintained exclusively with periodical sowing of mixed herbaceous plants (especially belonging to the families of Leguminosae and Cruciferae), and with distribution of organic matter (cow manure), produced internally, and composted according to biodynamic indications. The farm also grows pollinating insects (bees) to assist the pollination process. Pest management is performed mainly using horn-based biodynamic preparations, or with organic products [18].

To reduce the statistical error due to fruit variability which is expected in open field studies on apple trees [19] and to achieve an higher grade of accuracy in the determination of the effect of farming system on fruit physicochemical characteristics and chemical composition, a selection of seven apples showing the most similar size, shape, and skin colour was performed from each sample, and used for the following analyses.

Colour and physiochemical fruit parameters

The following parameters were obtained on seven selected fruits from each orchard: fresh weight (g), dimension (diameter and maximum height, mm), colour of skin and pulp as fresh and after starch iodine test (*L, a*, and *b* coordinates), and chroma index [calculated as $(a^2 + b^2)^{1/2}$]. Starch iodine test (Lugol solution) was performed by visual evaluation on halved fruits and by scoring samples on a 'Golden Delicious' standardized 1–9 scale [20]. Single fruits were tested for pulp firmness with an 11.3 mm plunger (Newton) hand penetrometer (Turoni, Italy) and with an Atago N1 (Atago Co., Tokyo, Japan) refractometer (°Brix) for soluble solids content (SSC). Titratable acidity (mg of malic acid/L) and pH were determined on 15 g of smashed pulp in 150 mL distilled water. For further details on colour and physiochemical parameters detection, see Taiti and colleagues [21].

Polyphenols content

Samples of 5 and 100 g finely powdered frozen skin and pulp were extracted with 25 and 250 mL, respectively, of

70% ethanol adjusted to pH 3.2 with formic acid at room temperature for 2 h using an ultrasonic bath. Seven replicates were obtained for each orchard. The hydroalcoholic extracts were analyzed by HPLC/DAD/MS for the determination of their polyphenol content. Authentic standards of rutin, epicatechin, and chlorogenic acid were purchased from Sigma–Aldrich (St. Louis, USA). All solvents were of HPLC-grade purity (BDH Laboratory Supplies, United Kingdom). Analysis of polyphenols was carried out using an HP 1100 L liquid chromatograph equipped with a DAD detector and managed by an HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA). The column was a 150 x 2 mm i.d. 3 µm Luna C18 (Phenomenex) operating at 27 °C. UV/Vis spectra were recorded in the 190–600 nm range, and the chromatograms were acquired at 280, 330, and 350 nm. The mobile phase was a four-step linear solvent gradient system, starting from 95% H_2O (adjusted to pH 3.2 by HCOOH) up to 100% CH_3CN during a 40 min period and a flow rate of 0.2 mL min^{-1}.

The HPLC system was interfaced with an Agilent TOF MS equipped with an ESI source (Agilent Corp, Santa Clara, CA, USA). The TOF/MS analysis was performed using the full-scan mode with the mass range set to *m/z* 100–1500 in both positive and negative modes. The conditions of the ESI source were as follows: drying gas, high purity nitrogen (N_2); drying gas temperature, 350 °C; drying gas flow-rate, 6 L min^{-1}; nebulizer, 20 psi; capillary voltage, 4000 V (negative) 4000 V (positive); fragmentation, 200 V; and skimmer, 60 V. The acquisition and data analysis were controlled using the Agilent LC-MS TOF Software (Agilent, USA).

Quantification of individual compounds was directly performed by HPLC/DAD using a five-point regression curve ($r^2 \geq 0.998$) in the range 0–30 mg on the basis of authentic standards. In particular, quercetin derivatives were determined at 350 nm using rutin as reference compound, catechins were determined at 280 nm using epicatechin as reference compound, and hydroxycinnamic derivatives were determined at 330 nm using chlorogenic acid as reference compound. In all cases, actual concentrations of the derivatives were calculated after applying, where possible, corrections for differences in molecular mass. The identity of polyphenols was ascertained using data from HPLC/DAD and HPLC-TOF analyses, by comparison with bibliographic data and combination of retention times, UV/Vis, and mass spectra with those of authentic standards.

PTR-ToF-MS analysis

The measurement of VOCs emitted by different samples was performed with a PTR-TOF 8000 instrument (Ionicon Analytik GmbH Innsbruck, Austria) using H_3O^+ as reagent ion for the proton transfer reaction (see Ellis and Mayhew [22] for an in-depth description of the technology). Measurements were performed at controlled temperature (20 ± 3 °C) and humidity (65%) to avoid any interference on VOCs emission and detection by such parameters [21].

According to Farneti et al. [15], samples were analyzed as cut flesh portions, as this has been found to be the most suitable way to analyze the aroma of apples and in a manner closer to human appreciation. In detail, each fruit was cut into four parts; a cube (3 cm^3) from each section was placed in a 250 mL glass vials provided with inlet and outlet Teflon pipes, which are connected, respectively, to a zero-air generator and to the PTR-ToF-MS system. Mass spectra from 20 to 220 *m/z* were recorded, using the following operating parameters in the drift tube: pressure 2.3 mbar, temperature 50 °C, voltage 600 V, and extraction voltage at the end of the pipe (Udx) 35 V, corresponding to an *E/N* value of 140 Td. The VOCs in the headspace were measured by direct injection into the drift tube inlet. Measurements on an empty vial were run before every sample measurement and used for background subtraction.

Raw data were acquired with the TofDaq software (Tofwerk AG, Switzerland) using a dead time of 20 ns for the Poisson correction; and peak extraction followed the methodology described in Taiti et al. [21], based on a modified Gaussian peak shape. PTR-ToF-MS spectra calibration was performed offline, using the peaks of known components, present in the spectra at any time (water isotope, $H_3{}^{18}O^+$, $m/z = 21.022$; nitric oxide, NO^+, $m/z = 29.99$; acetone, $C_3H_7O^+$, $m/z = 59.05$). All data from each replicate and background signal were normalized, according to Infantino et al. [23], by the primary ion signal (cps to ncps). Data were filtered, all peaks ascribed to water chemistry or other interfering ions (e.g., oxygen and nitrogen monoxide) were removed, and subsequently, signals whose concentration was lower than 0.5 ncps were discarded. Each measurement was obtained by the average of three recordings lasting 100 cycles, which correspond to 100 s/sample. Hence, data were normalized to sample mass (expressed in grams) for a better comparison between samples. Finally, the four recordings obtained on each fruit were reduced to two (by averaging data of opposite section). This procedure was performed on seven fruits for each orchard, for a total of 42 (14 × 3) mass spectra, on which statistical analysis was performed.

Statistical analysis

Principal component analysis (PCA) was used as unsupervised multivariate technique to represent and explore samples and variables correlations. Missing data were estimated using the mean of the corresponding variables. Pearson's correlation coefficient *r* as a statistical measure of the strength of a linear relationship between paired variables

and 95% confidence ellipses were also calculated. When applicable, comparisons of the means were performed with one-way ANOVA and Tukey's post-hoc test using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Where possible, the results are shown as mean ± SD interval.

Results and discussion

Colour and physiochemical fruit parameters

In general, the analysis of colour and physiochemical parameters of apples deriving from different geographical regions and agronomic management did not show very relevant differences. This agrees with other studies performed comparing apples grown in the conventional and organic farming (see Roussos and Gasparatos [24]), and the agronomic technique with which biodynamic management shares the highest number of similarities, including a reliance on organic fertilizers. In fact, for many of the parameters acquired (e.g., fruit weight and height, SSC, pH, and pulp colour), no significant differences were found. Nevertheless, from the comparative analysis of other parameters, some interesting dissimilarities could be underlined. For example, the shape and weight of the fruits from conventional orchard in Tuscany (CON-B) showed the highest diameter, while apples grown with similar procedures in Trentino Alto Adige (CON-A) showed the lowest (Table 1) ($p \leq 0.05$). In terms of the colour analysis, biodynamic apples (BIO-A) showed the brightest skin ("*L*" index, $p \leq 0.05$), while no significant differences were observed for pulp colour. Colorimetric measurement after Lugol treatment highlighted statistical differences for the "*b*" coordinate and the chroma index of pulps, showing a darker blue pulp for BIO-A and CON-B in respect to CON-A ($p \leq 0.05$). Accordingly, in the standardized maturation scale for 'Golden Delicious' apples, CON-A apples showed the highest starch hydrolysis grade (7–8) with respect to BIO-A (4–5) and CON-B (3–4). Such results were in agreement with the highest titratable acidity found in BIO-A and CON-B pulps in respect to CON-A ($p \leq 0.05$). CON-A fruits, notwithstanding its high value on the ripeness scale (7–8), showed the highest pulp firmness, with a statistically different value ($p \leq 0.05$) from the other samples. No significant differences were observed for SSC and pH of the juice.

Polyphenol content

Apple fruits (skin and pulp) were analyzed for their polyphenol content by chromatographic and spectrophotometric analyses. The mass spectra were obtained in both positive and negative modes by HPLC-TOF analysis, and the results achieved did not differ to previous studies (see Yuri et al. [10] and Chinnici et al. [25]). Table 2 lists polyphenol content of skin and pulp of apples grown in two contexts and under different agricultural management techniques. As expected, most of polyphenols were found in the skin, and the amount of flavonols in the pulp was about 0.2–0.6% of that of skin, hydroxycinnamic acids ranged from 6 to 15%, and catechins were from 16 to 24%. Skin and pulp polyphenol contents of 'Golden Delicious' apples were of the same magnitude order as what was previously reported [25]. Taking into account the different secondary metabolites under the two management conditions, only chlorogenic acid content seemed to characterize biodynamic grown apples; its amount was the highest in both skin and pulp ($p \leq 0.05$). On

Table 1 Colour and physiochemical fruit parameters in apples from different geographic regions and agronomic management

	BIO-A	CON-A	CON-B
Fresh weight (g)	224.8 ± 24.6	231.5 ± 43.6	231.4 ± 14.1
Diameter (mm)	79.0 ± 3.1^{a,b}	77.6 ± 5.4^{a}	81.6 ± 1.9^{b}
Height (mm)	78.6 ± 4.0	79.2 ± 7.5	75.9 ± 3.1
Firmness (N)	69.6 ± 3.9^{a,b}	75.5 ± 5.8^{b}	64.7 ± 6.8^{a}
SSC (°Brix)	14.7 ± 1.0	12.8 ± 1.8	13.3 ± 0.5
pH	3.4 ± 0.1	3.5 ± 0.1	3.4 ± 0.2
Titratable acidity (g/L)	2.3 ± 0.4^{b}	1.5 ± 0.5^{a}	2.3 ± 0.6^{b}
Skin colour (*L*)	80.1 ± 2.2^{b}	75.9 ± 2.3^{a}	74.7 ± 2.8^{a}
Pulp colour (*L*)	83.5 ± 1.0	81.8 ± 1.6	80.9 ± 2.7
Lugol test—pulp colour (*b*)	19.0 ± 8.8^{a}	30.4 ± 6.9^{b}	17.3 ± 4.6^{a}
Lugol test—ripeness (1–9 scale)	4–5	7–8	3–4

Titratable acidity is in g of malic acid/L of fresh juice. Data ± SD are shown; data marked by different letters within the same line are significantly different ($p \leq 0.05$)

BIO-A biodynamic apples grown in the location *A* Trentino Alto Adige; *CON-A* conventional apples grown in the location *A* Trentino Alto Adige; *CON-B* conventional apples grown in the location *B* Tuscany

Table 2 Polyphenols content, data are expressed as µg/g apple skin and pulp (fresh weight)

Compound		CON-A	BIO-A	CON-B
Apple skin				
Flavonols				
Q-rhamno-glucoside	H1-S	$42.4 \pm 9.4^{a,b}$	16.6 ± 6.0^{a}	65.4 ± 16.1^{b}
Q-galactoside	H2-S	$427.8 \pm 99.0^{a,b}$	280.4 ± 67.6^{a}	552.6 ± 109.8^{b}
Q-glucoside	H3-S	118.6 ± 48.6^{a}	84.0 ± 16.8^{a}	226.5 ± 29.6^{b}
Q-xyloside	H4-S	99.4 ± 9.4^{b}	79.5 ± 1.7^{a}	78.3 ± 4.8^{a}
Q-arabinopyranoside	H5-S	12.4 ± 2.3	17.2 ± 6.5	15.3 ± 1.3
Q-arabinofuranoside	H6-S	216.5 ± 9.5^{b}	173.8 ± 33.2^{ab}	161.0 ± 10.0^{a}
Q-rhamnoside	H7-S	211.8 ± 14.8^{b}	111.7 ± 13.4^{a}	90.6 ± 14.5^{a}
Total flavonols	H8-S	$1128.9 \pm 173.3^{a,b}$	763.2 ± 132.6^{a}	1189.8 ± 83.8^{b}
Caffeic acid derivatives				
Chlorogenic acid	H9-S	52.8 ± 2.3^{a}	87.9 ± 12.0^{b}	61.7 ± 9.7^{a}
Total catechins	H10-S	899.8 ± 81.2^{b}	$791.2 \pm 68.9^{a,b}$	611.6 ± 83.8^{a}
Apple pulp				
Flavonols				
Q-xyloside	H1-P	0.8 ± 0.1	0.9 ± 0.1	n.d.
Q-arabinopyranoside	H2-P	0.9 ± 0.1	1.0 ± 0.2	n.d.
Q-arabinofuranoside	H3-P	2.9 ± 0.5^{b}	3.4 ± 1.0^{b}	0.8 ± 0.1^{a}
Q-rhamnoside	H4-P	n.d.	n.d.	2.0 ± 0.6
Total flavonols	H5-P	4.6 ± 0.5^{b}	5.1 ± 0.9^{b}	2.8 ± 0.7^{a}
Caffeic acid derivatives				
Chlorogenic acid	H6-P	70.0 ± 6.5^{a}	102.8 ± 8.5^{b}	64.2 ± 17.3^{a}
p-coumaroyl-quinic acid	H7-P	10.8 ± 1.2^{b}	12.4 ± 1.7^{b}	7.6 ± 0.5^{a}
Total caffeic acid deriv.	H8-P	80.8 ± 7.7^{a}	115.2 ± 10.1^{b}	71.8 ± 17.6^{a}
Total catechins	H9-P	145.8 ± 8.0	149.0 ± 27.7	148.6 ± 32.3

Data ± SD are shown, data marked by *different letters* within the *same line* are significantly different ($p \leq 0.05$), *n.d.* not determined, *Q* quercetin

BIO-A biodynamic apples grown in the location *A* Trentino Alto Adige; *CON-A* conventional apples grown in the location *A* Trentino Alto Adige; *CON-B* conventional apples grown in the location *B* Tuscany

the other hand, total skin flavonol content was the lowest in biodynamically grown apples.

In many experiments with different apple cultivars, no differences have been found between organic and conventional managements [10]. In the case of 'Golden Delicious' apples, a higher amount of total phenolics in organically grown fruits with respect to integrated production was found [11]. For the same cultivar in a 3-year study, the differences among years were more relevant than those between organic and conventional farming [9]. Higher values of chlorogenic acid were found in organically cultivated 'Golden Delicious' apples with respect to integrated cultivation [11], although different comparative results were obtained when the sampling was performed for more than 1 year [9].

Principal component analysis on HPLC data

PCA performed on skin and pulp data set derived from the polyphenol analysis by HPLC/DAD/MS (Table 2) is represented in Fig. 1 (top right and left). The first two components obtained with PCA explained more than 76% of the total variability and the derived two-dimensional plot provided the separation of the three samples (Fig. 1, top left). The correlation plot (Fig. 1, top right) showed strong positive correlations between certain flavonols. In fact, concerning the skin analysis, two compact groups could be found: that of Q-rhamno-glucoside (H1-S), Q-galactoside (H2-S), and Q-glucoside (H3-S) ($r = 0.907$) and that of Q-xyloside (H4-S), Q-arabinofuranoside (H6-S), and Q-rhamnoside (H7-S) ($r = 0.805$). Similarly, for the pulp analysis, Q-xyloside (H1-P), Q-arabinopyranosid (H2-P), and Q-arabinofuranoside (H3-P) were found well linked ($r = 0.957$). All caffeic acid derivatives showed positive correlation (although not statistically significant, $r = 0.603$); interestingly, some of them were negatively correlated to some flavonol compounds, namely, the caffeic acid derivatives of the pulp and Q-rahmno-glucoside (H1-S), Q-galactoside (H2-S), and Q-glucoside (H3-S) of the skin ($r = -0.617$). Further information could be

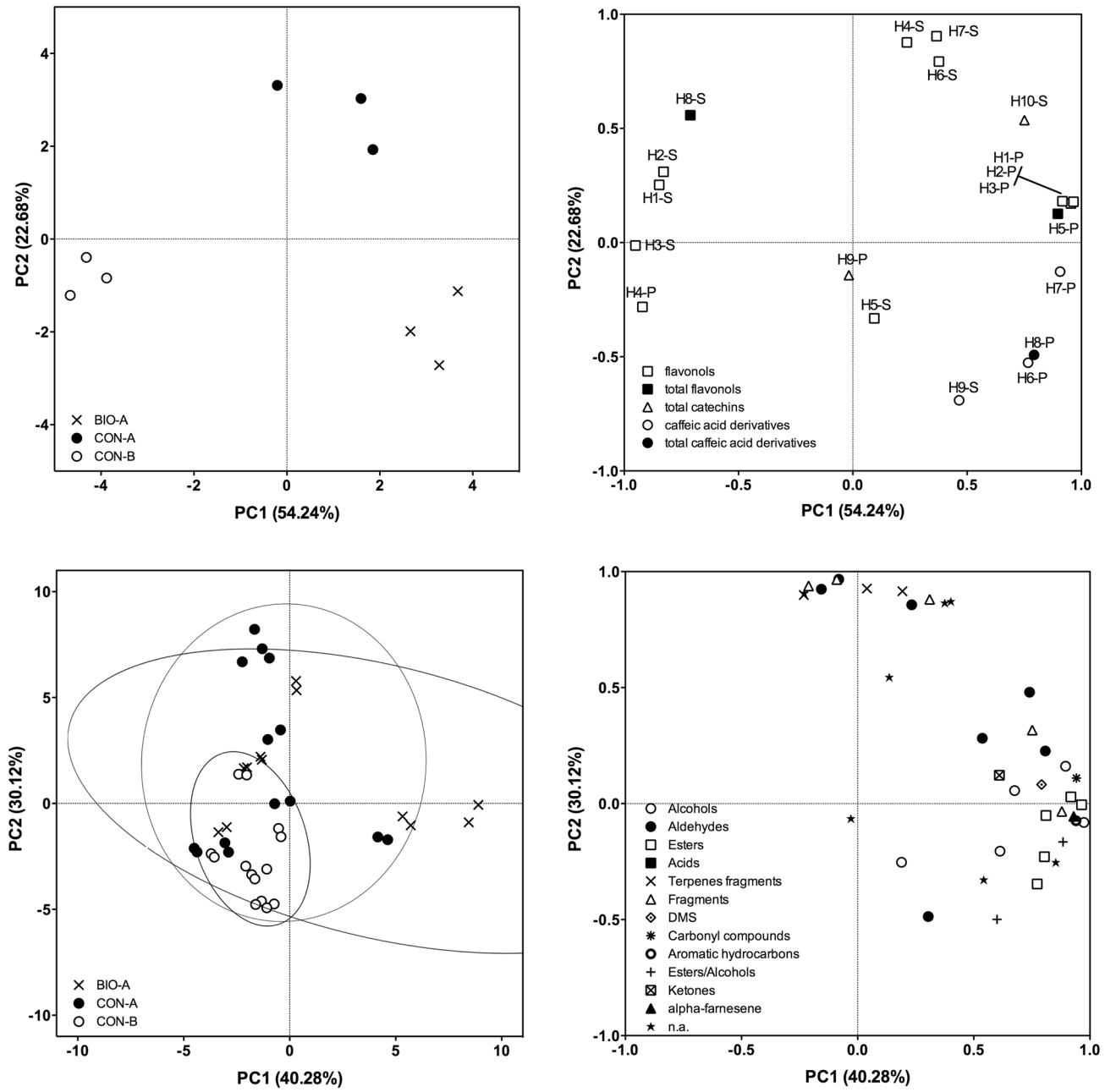

Fig. 1 *Biplots* showing the projection of the samples in the two-dimensional space (*left*) and correlation biplots of the variables are shown (*right*). When possible, samples are grouped into classes (95% confidence *ellipses*). The analysis was applied to 19 parameters (HPLC/DAD/MS analysis, *top*) and to 39 parameters (PTR-TOF-MS analysis, *bottom*). *S* skin, *P* pulp, *BIO-A* biodynamic apples grown in the location *A* Trentino Alto Adige, *CON-A* conventional apples grown in the location *A* Trentino Alto Adige, *CON-B* conventional apples grown in the location *B* Tuscany

achieved by superimposing the plots of samples and correlations. For example, four variables obtained by HPLC/DAD/MS analysis performed on skin, Q-rhamno-glucoside (H1-S), Q-galactoside (H2-S), Q-glucoside (H3-S), and total flavonols (H8-S), shared the same area (i.e., the negative range of PC2) with apples grown in Tuscany (CON-B) with conventional agronomic management; in the skin of such fruits, in fact, the highest content of those compounds was found.

Among the original variables that contributed to the PCs, it is worth noting that the first PC (that described 54.24% of variability) was defined mainly by analytical parameters obtained from the analysis of pulp, especially flavonols (Table 4), while parameters obtained from the

Table 3 Masses found by PTR-ToF-MS analysis in the apple samples

	Measured mass	Theoretical protonated mass	Protonated formula	Chemical class	Tentative identification	References
M1	27.023	n.a.	n.a.	n.a.	n.a.	n.a.
M2	31.017	31.034	CH_3O^+	Aldehyde	Formaldehyde	[15]
M3	33.034	33.009	CH_5O^+	Alcohol	Methanol	[16, 21]
M4	39.022	39.057	$C_3H_3^+$	n.a.	n.a.	[15]
M5	41.037	41.038	$C_3H_5^+$	Fragment	Fragment of alcohol/ester/alkyl	[15, 21]
M6	43.017	43.045	$C_2H_3O^+$	Ester	Esters	[15]
M7	43.054	43.089	$C_3H_7^+$	Alcohol	Alcohols	[15]
M8	45.033	45.0334	$C_2H_5O^+$	Aldehyde	Acetaldehyde	[15, 16, 21, 27–29]
M9	47.010	n.a.	n.a.	n.a.	n.a.	[15]
M10	47.049	47.075	$C_2H_7O^+$	Alcohol	Ethanol	[13, 16, 21, 27–32]
M11	53.038	53.084	$C_4H_5^+$	n.a.	n.a.	[15]
M12	55.054	55.100	$C_4H_7^+$	n.a.	n.a.	[15]
M13	57.033	57.072	$C_3H_5O^+$	Fragment	Aldehydes (hexanal/decanal), ester (methyl 2-methyl butanoate/ethyl 2-methyl butanoate/ butyl propionate/hexyl acetate/propyl propanoate)	[15]
M14	57.069	57.116	$C_4H_9^+$	Alcohol	(1-butanol/1-pentanol/1-hexanol,2-methyl-1-propanol/pentanol/iso-pentanol/1-heptanol/2-ethyl-1-hexanol/octanol/nonanol)	[15, 33]
M15	59.049	59.029	$C_3H_7O^+$	Aldehydes/ketones (carbonyl compound)	Propanal/2-propanone/acetone	[15, 16, 21, 27–29, 33, 34]
M16	61.028	61.018	$C_2H_5O_2^+$	Ester/acid	Esters (ethyl acetate/butyl acetate/2-methylbutyl acetate/hexyl acetate/propyl acetate/isubutyl acetate/isoamyl acetate/amyl acetate), acetic acid	[15, 26, 27, 31, 35]
M17	63.027	63.136	$C_2H_7S^+$	Organosulfur compound	Dimethylsulfide (DMS)	[27, 34, 36]
M18	67.054	n.a.	n.a.	Terpenes fragment	Terpenes	n.a.
M19	69.069	69.127	$C_5H_9^+$	Terpenes fragment	α-Farnesene	[15, 33]
M20	71.049	71.099	$C_4H_7O^+$	Ester fragments	Ethyl butanoate/ethyl hexanoate/propyl butanoate/2-methylbutyl acetate/isoamyl acetate	[15]
M21	71.085	71.143	$C_5H_{11}^+$	Alcohol	2-Methyl-butanol/pentanol/iso-pentanol/2-ethyl-1-hexanol/octanol/nonanol	[15]
M22	73.064	73.039	$C_4H_9O^+$	Aldehyde	Butanal	[13, 15, 16, 28, 33]
M23	75.040	75.028	$C_3H_7O_2^+$	Ester	Butyl propanoate/methyl acetate	[15, 29, 37, 38]
M24	79.039	79.075	$C_2H_7O_3^+$	n.a.	n.a.	[15]
M25	81.069	81.138	$C_6H_9^+$	Terpenes fragment	Terpenes	[15]
M26	83.085	83.154	$C_6H_{11}^+$	Fragment	Alcohols ((Z)-3-hexen-1-ol/(E)-2-hexen-1-ol/5-hexen-1-ol), aldehyde (hexanal)	[15]

Table 3 (continued)

	Measured mass	Theoretical protonated mass	Protonated formula	Chemical class	Tentative identification	References
M27	85.064	85.126	$C_5H_9O^+$	Fragment	Ester (hexyl acetate), acid (2-methyl butanoic acid)	[15]
M28	85.101	n.a.	n.a.	n.a.	n.a.	n.a.
M29	87.044	87.038	$C_4H_7O_2^+$	Ketone	Lactone (butyrolactone)	[13, 15]
M30	87.080	87.049	$C_5H_{11}O^+$	Aldehyde/alcohol/ether	Aldehides (3-methylbutanal/pentenal/2-methylbutanal/3-methylbutanal/pentanal)/ alcohol (1-pentanol/2-methyl-2-buten-1-ol//3-penten-2-ol)/ether (allyl ethyl ether)	[13, 16, 28–30, 32, 37–40]
M31	89.059	89.038	$C_4H_9O_2^+/C_5H_{13}O^+$	Ester/alchol	Ester (ethyl acetate/ethyl butanoate/propyl butanoate/methyl propionate), alcohol (3-methylbutanol	[13, 15, 16, 28–32, 37, 38, 41]
M32	97.064	97.059	$C_6H_9O^+$	Aldehyde	2,4-Hexadienal	[28]
M33	99.080	99.05	$C_6H_{11}O^+$	Aldehyde/ester	Aldehydes (trans-2-hexenal/(E)-2-hexenal), esters (ethyl hexanoate/hexyl acetate)	[13, 15, 28, 35, 41]
M34	101.096	101.059	$C_6H_{13}O^+$	Aldehyde	Hexanal	[13, 15, 16, 28, 29, 37–39, 41]
M35	103.075	103.048	$C_5H_{11}O_2^+/C_6H_{15}O^+$	Ester/alcohol	Esters (methylbutanoic acid/ethyl 2-methyl butanoate/propyl acetate/ethyl propionate), alcohols (1-hexanol)	[13, 15, 16, 28, 29, 31, 32, 35, 37–39]
M36	115.112	115.196	$C_7H_{15}O^+$	Aldehyde	Heptanal	[16, 29]
M37	117.091	117.058	$C_6H_{13}O_2^+$	Acid/ester	Hexanoic acid/isobutyl acetate/methyl 2-methyl butanoate/ethyl butanoate/ethyl hexanoate/ butyl acetate/isubutyl acetate/propyl propanoate	[13, 16, 28, 30–32, 35, 39]
M38	121.064	121.09	$C_9H_{13}^+$	Aromatic hydrocarbon	1,3,5-Trimethylbenzene	[31]
M39	149.100	149.099	$C_{10}H_{13}O^+$	Terpenoid/terpenoid fragment	Terpenoids (estragole)/α-farnesene	[13, 15, 16, 29, 31, 33, 39]

When applicable, tentative identification is based on comparisons with previous published investigations; n.a. (not applicable) is reported for those masses detected but not yet described in the literature

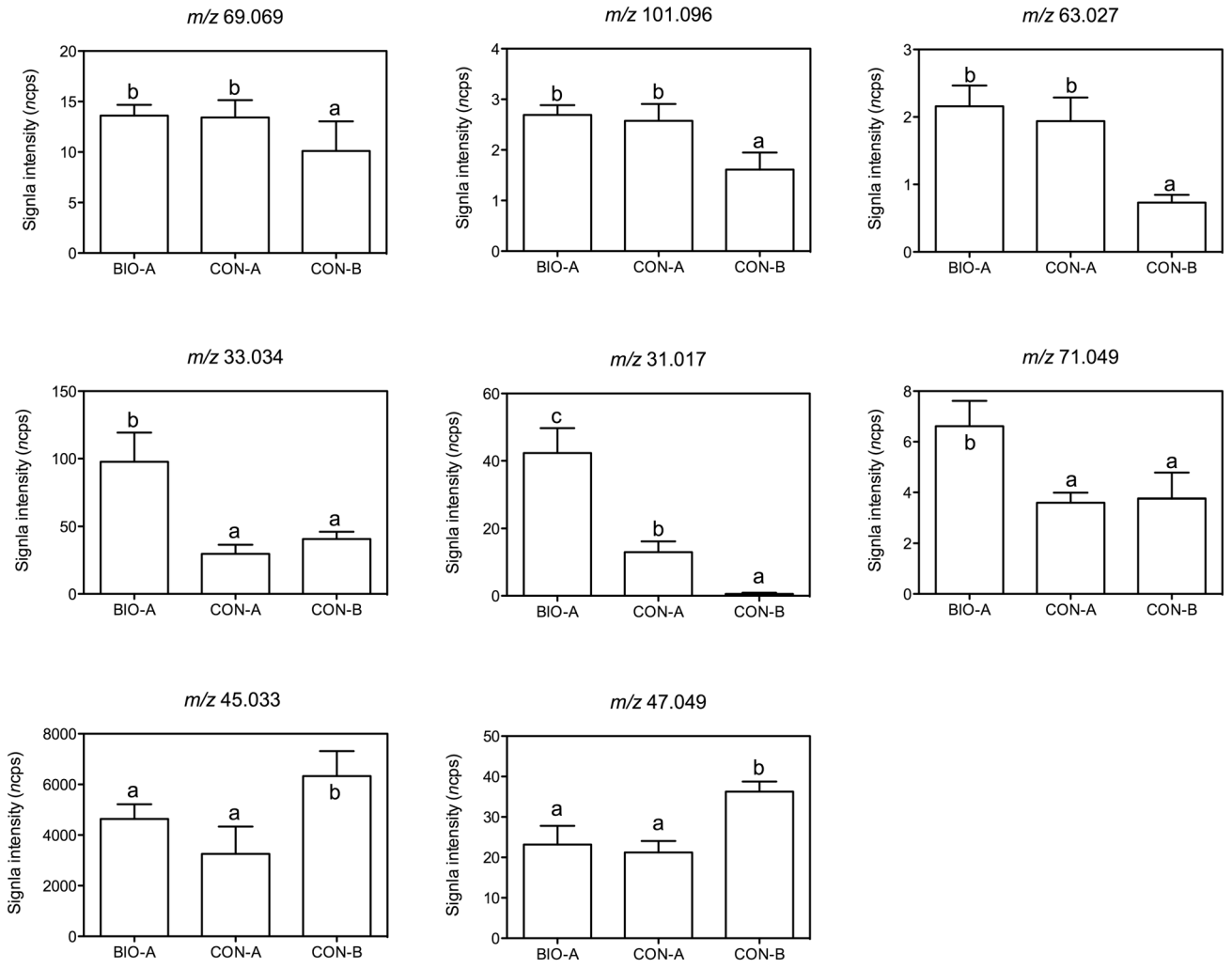

Fig. 2 VOCs intensities for some for some compounds showing differences among samples: from *top left* to *bottom right*: fragment of α-farnesene (M19), hexanal (M34), DMS (M17), methanol (M3), formaldehyde (M2), esters (M20), acetaldehyde (M8), and ethanol (M10). Data ± SD are shown; data marked by *different letters* within the *same line* are significantly different ($p \leq 0.05$)

skin contributed to the definition of the second PC, especially caffeic acid derivatives.

PTR-ToF-MS spectra analysis

The analysis performed on the PTR-ToF-MS mass spectra of the headspace of the three groups of samples under study allowed the compilation of a table of 39 mass peaks. Table 3 shows all masses highlighted by PTR-ToF-MS analysis and their possible identification, taking into account the available fragmentation patterns of pure standards (see Masi et al. [26] for references). Most of the masses listed have already been identified in apples using PTR-MS approach (see references listed in Table 3). The same approach was not possible for other few masses (referred as n.a., not applicable). An equal number to any possible compound having the same protonated theoretical mass were attributed. In two cases (*m/z* 89.038 and *m/z* 89.049 for M31; *m/z* 103.048 and *m/z* 103.059 for M35), different protonated theoretical masses of putative compounds have been associated: in both cases, the system detected only one peak due to the small differences in their mass.

In general, the three sets of samples showed quite similar VOCs profiles in terms of signal intensity, suggesting that VOCs are determined more by their genetic regulation than by external factors [42]. Nevertheless, some interesting differences could be underlined, especially for apples grown in different geographical regions, whatever the cultivation technique (Fig. 2). In fact, apples grown in Trentino Alto Adige showed higher VOCs intensities, especially for terpenes (e.g., *m/z* 69.069), many aldehydes (e.g., hexanal, *m/z* 101.096), and dimethylsulfide (DMS, *m/z* 63.027) (Fig. 2, top). Concerning terpenes, the tentative identification performed pointed the attention on two masses (*m/z*

Table 4 Variables that contribute most to the description of sample variability as underlined by principal component analysis (PCA)

HPLC/DAD/MS data		PTR-ToF-MS data	
Variables contributing to PC1 (%)			
H2-P	9.045	M7	6.039
H1-P	8.829	M23	5.911
H3-S	8.776	M38	5.625
H4-P	8.212	M14	5.648
H3-P	8.156	M39	5.500
Variables contributing to PC2 (%)			
H7-S	18.985	M19	7.319
H4-S	17.851	M32	7.968
H6-S	14.585	M13	7.938
H9-S	11.098	M33	7.487
H8-S	7.221	M36	7.260

The analysis was applied to 19 parameters (HPLC/DAD/MS analysis; *S* skin; *P* pulp) and to 39 parameters (PTR-ToF-MS analysis). Here, we select the first five variables with highest influence for the first two components (PC1 and PC2), namely, with squared cosine bigger than 0.59. Mass is numbered according to Tables 2 and 3

149.100, M39 *m/z* 69.069, M19), tentatively identified as α-farnesene and its fragments, respectively. The terpenoid α-farnesene is linked to fruity, citric, and floral aroma descriptors, contributing largely to the typical green apple aroma. Together with esters, α-farnesene is one of the most characteristic and abundant volatile compounds emitted by apples [13] and has been proposed for cultivar classification [40]. This terpenoid is normally found in stored apples [39], and it is expected to increase significantly with post-harvest ripening, starting from the first few days [17]. Other authors [15, 16, 33] have tentatively identified *m/z* 149.100 as estragole, a terpenoid ether associated with a spice-like or aniseed aroma in apple, mainly produced during post harvest maturation as well. Interestingly, terpenoids, linked to fruits storage and abundant since the very first days after harvest, were completely absent in apples from Tuscany (CON-B) and low in the other samples (data not shown); the high signal intensity found for *m/z* 69.069 (M19), identified as a fragment of α-farnesene (Fig. 2, top left), suggests that fragmentation process occurred. Hexanal (*m/z* 63.027, M17), described as green apple-like odours, is associated with the aroma intensity of apple fruits, especially 'Golden Delicious' apples [41]; thus, according to our results, apples grown in Trentino Alto Adige had higher aroma intensity that those grown in Tuscany (Fig. 2, top central). DMS is a very common compound in food; in most cases, sulfur compounds confer appreciated flavour characters only when present at low concentrations; for example, in apple juice, its elevated presence is linked to poor sensory scores [36]. In our samples, DMS intensity is generally low, especially in apples obtained by the conventional cultivation procedures performed in Tuscany (CON-B), whose mean value was statistically lower to that of apples from Trentino Alto Adige, whatever the agronomic technique adopted (Fig. 2, top right).

Besides those compounds found in higher amounts in apples from Trentino Alto Adige than in those from Tuscany, it is worth noting that apples produced using a biodynamic approach (BIO-A) showed the highest intensity for many compounds in the classes of alcohols (e.g., *m/z* 33.034, identified as methanol, Fig. 2, middle left), aldehydes (e.g., *m/z* 31.017, identified as formaldehyde, Fig. 2, middle central), and esters (e.g., *m/z* 71.049, identified as ethyl butanoate or ethyl hexanoate or propyl butanoate or 2-methylbutyl acetate or isoamyl acetate) (Fig. 2, middle right), which are recognized to be fundamental in determining apple aroma [43] and that usually predominate in ripe apples [37]. On the other hand, apples obtained with the conventional techniques in Tuscany (CON-B) showed lower intensities for almost all detected compounds, with the exception for M8 (*m/z* 45.033), tentatively identified as acetaldehyde (Fig. 2, bottom left), and M10 (*m/z* 47.049), identified as ethanol (Fig. 2, middle central), which are typically produced during the ripening process. In fact, acetaldehyde accumulates during ripening, even under aerobic conditions, and it is then transformed in ethanol and acetyl coenzymeA (CoA), leading subsequently to the production of several esters [15]. Such results may be in contrast with those underlined by the analysis of colour and physiochemical parameters, where CON-B apples appeared to be in an early ripeness stage.

Principal component analysis on PTR-ToF-MS data

By performing a PCA on 39 mass peaks detected by PTR-ToF-MS (Table 3), two new components turned out to

explain about 70% of the total variability and the derived two-dimensional scatter plot (Fig. 1, top left) showed the three groups not clearly separated. Nevertheless, apples from Tuscany (CON-B) formed a fairly compact group, positioned in the negative range of both axes. Apples from Trentino Alto Adige, whatever the agronomic technique, formed much less compact groups, occupying also the positive range of both PC1 and PC2 axes. These results agree with those reported by Granato and colleagues [44] on the inability, using a PCA applied to PTR-ToF-MS data, to differentiate organic and biodynamic grape juices from conventional ones; they also confirm that, in apple fruits, volatile compounds are highly genetically regulated and stable across different environments [42].

Further interesting information could be achieved from the analysis of the contribution of each original variable to the new ones generated with the PCA; in Table 4, the first five most informative variables are reported for the first two principal components (PC1 and PC2). The compound *m*/*z* 43.054 (M7, tentatively identified as an alcohol compound) played the biggest role in the definition of the first component of PCA, describing 40.28% of total variability. Among the masses that contributed to PC1, the two compounds *m*/*z* 75.040 (M23) and *m*/*z* 121.064 (M38), respectively, identified as an ester compound (perhaps butyl propanoate or methyl acetate) and an aromatic hydrocarbon (perhaps 1,3,5-trimethylbenzene), and were highlighted. Other compounds with high contribution on PC1 were *m*/*z* 57.069 and *m*/*z* 149.100 (M14 and M39), tentatively identified as an alcohol and the terpenoid estragole (also identified as a fragment of α-farnesene). Mass M14, possibly identified as 1-butanol, has been previously listed as a key compound for cultivar and geographical origin distinction in monovarietal apple juices [45].

A terpene fragment perhaps deriving from the fragmentation of α-farnesene [15] was also one of the main contributors to PC2 (M19, *m*/*z* 69.069). Other interesting compounds describing the variability among samples were two compounds tentatively identified as aldehydes and two compounds probably derived from the fragmentation of an aldehyde or an ester. The first two were *m*/*z* 97.064 (M32, perhaps 2,4-hexadienal) and *m*/*z* 115.999 (M36, perhaps heptanal); the fragments were *m*/*z* 57.033 and *m*/*z* 99.05 (M13 and M33, respectively). Mass M13 may be identified as a fragment of hexanal, and its high discriminant role has been reported by Gan and colleagues [45] for apple juices, both in terms of cultivar and geographical origin. The same mass could be tentatively identified also as butyl propionate, previously indicated as one of the key compounds positively influencing the acceptability of apple [37]. Mass M33, whose possibly identification comprise also hexyl acetate, is one of the ester compounds that predominates in 'Golden Delicious' apples [46] and is typically associated with fruity flavour; it has a strong power in discriminating among apple juices obtained from different apple varieties [45, 47].

As underlined in the correlation plot showing a projection of the initial variables in the PCs space (Fig. 1, bottom right), two main groups could be defined. The first group of masses, positioned in the positive range of PC1, was composed of a great number of variables, including all the esters, alcohols, acids, terpenes, and almost all identified aldehydes. These compounds proved to be generally well correlated. In particular, esters, acetaldehyde, and ethanol (two anaerobic metabolites) were positively correlated; however, the mean correlation value was not statistically significant ($r = 0.502$). The positive correlation between esters, aldehydes, and alcohol metabolites is expected during the ripening process and is fundamental for the production of aroma compounds [15]. The second group of masses was composed by fragments, few aldehydes, and other not identified compounds, which showed mean pairwise significant positive correlation ($r = 0.892$). All the VOCs tentatively identified as terpenes fragments belonged to this group and were positively correlated (mean $r = 0.602$), with the exception of α-farnesene/estragole which itself was linked to the first group.

Conclusions

In conclusions, information on polyphenols content was useful to clearly separate samples both on geographic and agricultural management basis, according to PCA analysis, while the analysis of volatile organic compounds could only be used to highlight specific differences in the set of samples. Indeed, the composition of secondary metabolites of crop products is highly affected by the plant physiopathological conditions as well as by external factors; accordingly, it is expected to be influenced also by the agronomic management. Biodynamic system, with limited use of pesticides, and based only on natural preparations, may have dramatically influenced the plant's investment into its own defence systems [48], which resulted in a different content of polyphenols, but not of volatile compounds, being the latter characterized by a stronger genetic stability across different environments [42].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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