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click for updatesCite this: *Anal. Methods*, 2014, 6, 7484Received 23rd April 2014
Accepted 7th July 2014

DOI: 10.1039/c4ay00971a

www.rsc.org/methods

Qualitative discrimination between organic and biodynamic Sangiovese red wines for authenticity

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In this study the ability of the droplet evaporation method (DEM) to discriminate between organic and biodynamic Sangiovese red wines was studied for the first time. The relationship between the sizing-parameters and shape-descriptors of the crystal structures and the chemical parameters of wines was evaluated, and the most significant correlations were found between the intensity of red color of wines with circularity ($r = 0.90$) and solidity ($r = 0.94$). Moreover the shape-descriptors allowed a discrimination between the organic and biodynamic Sangiovese red wines based on two kinds of structures: (i) needle-like and (ii) flower-like forms.

1. Introduction

The emerging concern about healthy foods has increased the worldwide interest in organic viticulture,¹ organic wine,² and the biodynamic approach^{3,4} due to their low environmental impact.⁵ In particular, biodynamic agriculture, developed by Rudolf Steiner (1861–1925), is a method of sustainable organic farming that differs from the traditional organic systems mainly in the use of several soils and plant amendments, called *preparations*, made from cow manure (no. 500), ground quartz silica (no. 501) and seven medicinal plants that are specific to produce biodynamic compost.^{4,6} Although some researchers found that the use of these preparations can enhance both soil and crop quality,⁷ there is little information about the effect of the biodynamic management of vineyard on the quality of wine.⁸

The authentication of functional foods & beverages is fundamental to certify the products and our recent attempt to discriminate between organic and biodynamic Sangiovese red wines by means of NMR revealed that the year of grape harvest and vinification protocol were the two main factors that affect the composition of wines, whereas the vineyard management seemed to have limited effect on the wine quality, mainly in terms of some aminoacids (proline, aspartic acid and valine), alcohols and some polyphenols.⁹

Common chemical analyses usually start at the whole product level and finish at the molecular level with the identification and quantification of compounds (*i.e.* top-down approach), however, the opposite bottom-up approach is also possible and it is currently performed by the droplet

evaporation method (DEM), an evaporative self-assembly technique, that depending on the characteristics of the evaporating solution can lead to the formation of different structures including crack-patterns,¹⁰ amorphous agglomerates,¹¹ films,¹² and polycrystalline structures of different shapes,¹³ that have proved to be useful for diagnostic purposes of biological liquids already.¹⁴

In this study the application of DEM as a tool for the discrimination of red wines from organic and biodynamic vineyards is presented for the first time.

2. Materials and methods

2.1. Grapes and winemaking protocol

The red grapes cultivar Sangiovese, clone FEDIT 30 Esave grafted on root-stock Kober 5BB and planted on 2003, were obtained from organic and biodynamic management during the harvest 2010 and 2011 in experimental vineyards located in the Emilia-Romagna region (Tebano, RA, Italy). The plantation spacing was 2.8×1 m corresponding to 3571 spur-pruned vines per hectare (ha). Organic management was carried out in accordance with Reg. EC 834/2007,¹ whereas the biodynamic management differed from the organic one for the use of biodynamic *preparations*, that were stirred and sprayed according to the biodynamic principles during the vegetative growth, as follows: soil-applied cow manure (500; 100 g ha^{-1}) and fladen (cow manure enriched with basalt powder and eggshell; 100 g ha^{-1}), and canopy-applied finely ground quartz powder (501; 5 g ha^{-1}).

The winemaking protocol was the same for all trials and was carried out in stainless steel tanks according to the Italian Association of Organic Agriculture (AIAB), as follows: the grapes (*ca.* 200 kg each trial) were hand harvested at optimum technological maturity based on total soluble solids ($^{\circ}\text{Brix}$) and

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acidity values, placed in plastic bins of *ca.* 20 kg and transported to the experimental winery for the vinification process that included (i) destemming and crushing of grapes, and (ii) addition of potassium metabisulphite (10 g hL^{-1}), nutrients complex Nutristart (30 g hL^{-1}) and selected dry yeasts F15 (20 g hL^{-1} *Saccharomyces cerevisiae*). The fermentation tank was homogenized once every day by plunging the cap (*i.e.* grape skins) into the wine. Alcoholic and malolactic fermentations were monitored with time by residual sugars and the malic acid

content, respectively, and after completion the wines were stabilized in a cold room ($-4 \text{ }^\circ\text{C}$) and then kept at $4 \text{ }^\circ\text{C}$ until chemical analyses.

The experimental design included 8 trials (*i.e.* samples): 2 vineyard managements (organic and biodynamic), 2 field replicates (one for each management), and 2 years (2010 and 2011).

2.2. DEM analysis of wines

2.2.1. Experimental protocol. The experimental procedure was adopted from our previous study.¹¹ Briefly, the wines were uncorked and left for 30 min at room temperature, then $3 \mu\text{l}$ drops were collected with a micropipette and poured onto a cleaned microscope slide ($9 \times 9 \text{ cm}$) that was placed in a thermostatic chamber at $25 \text{ }^\circ\text{C}$ until the drying was completed. Next, the dry residues of wine droplets were photographed with a trinocular laboratory microscope (MT4300H, MEIJI Techno, Saitama, Japan), connected to a CMOS Camera (UK1175-C, EHD imaging GmbH, Damme, Germany) in QXGA resolution (2048×1536 pixels) and magnifications $25\times$, $100\times$, and $400\times$. The analysis was repeated three times for each sample with a total of 120 droplet residues: 8 samples (2 managements, 2 field replicates/management, 2 years), 3 replicates, and 5 droplets/replicate.

2.2.2. Image analysis. The droplet residues photographed in magnification $25\times$ and dark field were converted to binary images and analyzed by means of the particle analysis tool

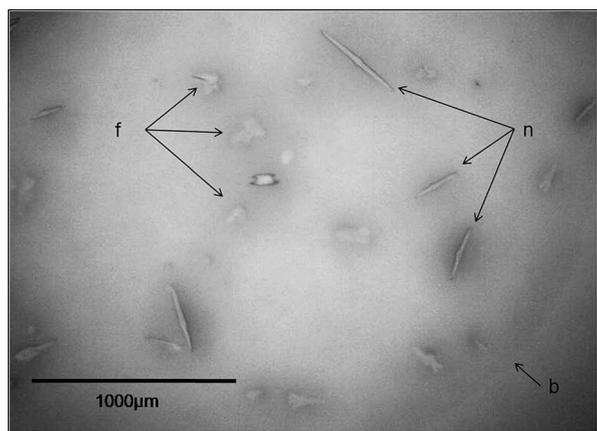


Fig. 1 Example of the evaporative residue of Sangiovese red wine droplet. The following pattern-elements can be observed: border (b), flower-like structures (f), and needle-like structures (n).

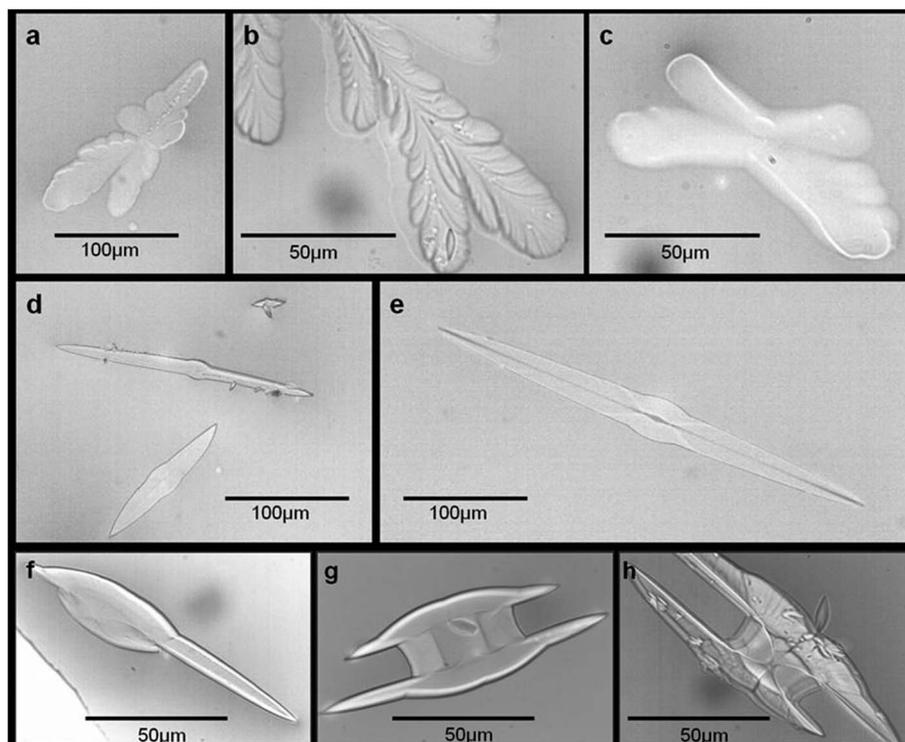


Fig. 2 Examples of the structures present in the evaporative residue of Sangiovese red wine droplet: (a–c) flower-like structures, (d and e) needle-like structures, and (f–h) form-evolution from a needle-like to a four-armed structure with two parallel arm-pairs.

ImageJ v. 1.47 (ref. 15) that allowed us to select both the sizing parameters and the shape descriptors, as follows:

- sizing parameters: count (number of structures), perimeter (length of the outside boundary of all the structures), total area (area of all the structures) and structure area;
- shape descriptors: circularity (C) and solidity (S) were calculated as follows:

$$C = \frac{4\pi A}{P^2}$$

where, A is the area of the structure, and P its perimeter. The value of 1.0 indicates a perfect circle, whereas a value close to 0.0 indicates an elongated shape.

$$S = \frac{A}{A_{\text{convex}}}$$

where, A is the area of the structure and A_{convex} is its convex area.

The intensity of the film color was considered another sizing-parameter and was measured on bright-field images in magnification $100\times$ in three rectangular regions of interest (ROI) per image. ROIs, variable in size to contain only structure-free areas, were analyzed by means of the command 'analyze/measure' for the mean pixel intensity (from 0 = black; to 255 = white).

2.3. Chemical analyses of wines

The following enological parameters were selected according to the Italian regulation of the Sangiovese di Romagna appellation wine¹⁶ and analyzed using the European Official Methods:¹⁷ ethanol content (% v/v), total dry extract (g l^{-1}), and optical density at 520 nm (red colored phenolic compounds). Besides

the above parameters, reducing sugars,¹⁸ total phenolic compounds¹⁹ and the pH of wines were also analyzed. All the parameters were analyzed for the 8 samples and the measurements were replicated 2 times.

2.4. Statistical analysis

The dataset was analyzed by means of analysis of variance (ANOVA) followed by the post-hoc multiple mean comparison with Turkey's Honestly Significant Difference test using the CoStat statistical software (v. 6.4, CoHort Software). In addition, the Bravais-Pearson linear coefficient of correlation $r = (\text{Cov}(X, Y))/(\text{SD}(X)\text{SD}(Y))$ was computed on the 8 samples to determine the degree of association between the DEM data (sizing parameters and shape descriptors of the needle-like and flower-like structures) and the chemical parameters of wines.

3. Results and discussion

3.1. Visual evaluation of DEM patterns

The residues of red wine droplets were composed of a droplet border and an inner space, entirely covered by a thin film, which contained seemingly random scattered structures of varying shapes (Fig. 1). Two structure types can be distinguished: (i) round, flower-like (Fig. 2a-c) and (ii) long needle-like forms (Fig. 2d and e). Each type showed distinct characteristics: (i) the flower-like forms grew from the structure-centre in different directions creating thick dendrites which resembled randomized fractals, whereas (ii) the needle-like forms grew from the structure-centre in only two opposite directions creating long, sharp-ended structures which often exhibited surprisingly exact symmetries (Fig. 2e). In some cases the needle-like structures evolved into four-armed structures with two parallel arm pairs (Fig. 2f-h). Moreover, the needle-like structures often contained secondary crystallizations and, in the biodynamic samples only, cracks perpendicular to the long axis (Fig. 3).

3.2. Computerized evaluation of DEM patterns

The results of computerized evaluation reflected the differences due to weather conditions (much rainfall in 2010, and warm summer in 2011) and plant treatments (vine bunches were thinned in 2011 only) (Tables 1 and 2). For what concerns the discrimination of wines from the two cultivation systems, the shape-descriptors of the pattern structures (*i.e.* circularity and solidity) were always significantly higher for the organic wines

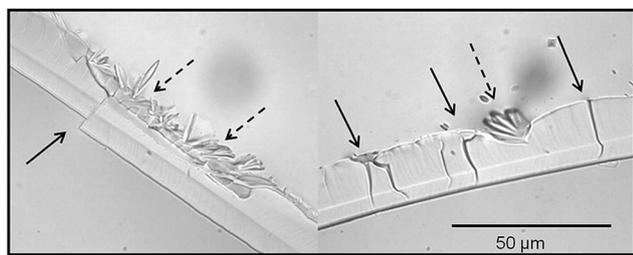


Fig. 3 Examples of the needle-like forms with cracks (continuous-line arrows) and crystallizations of secondary order (dashed-line arrows) typical of biodynamic Sangiovese red wine.

Table 1 Statistics of the structure sizing-parameters for organic and biodynamic wines per harvesting year^a

| Wine trial | N | Count | Total area [$\times 10^3$] | Structure area [$\times 10^3$] | Perimeter [$\times 10^3$] | Film intensity |
|-----------------|----|---------------------|------------------------------|----------------------------------|-----------------------------|-----------------|
| | | Mean \pm SE | Mean \pm SE | Mean \pm SE | Mean \pm SE | Mean \pm SE |
| Organic 2010 | 30 | 10.5 \pm 0.8 (ab) | 50 \pm 4 (a) | 4.8 \pm 0.2 (a) | 0.64 \pm 0.03 (a) | 192 \pm 3 (a) |
| Biodynamic 2010 | 30 | 11.9 \pm 0.7 (a) | 52 \pm 4 (a) | 4.4 \pm 0.2 (a) | 0.67 \pm 0.02 (a) | 179 \pm 3 (b) |
| Organic 2011 | 30 | 12.0 \pm 1.3 (a) | 43 \pm 4 (a) | 3.6 \pm 0.15 (b) | 0.48 \pm 0.02 (b) | 160 \pm 3 (d) |
| Biodynamic 2011 | 30 | 8.8 \pm 0.8 (b) | 29 \pm 3 (b) | 3.3 \pm 0.1 (b) | 0.46 \pm 0.02 (b) | 169 \pm 4 (c) |

^a Legend: N = number of samples; SE = standard error; different letters indicate significance at $p \leq 0.05$.

Table 2 Statistics of the structure shape-descriptors for organic and biodynamic wines per harvesting year^a

| Wine trial | N | Circularity | Solidity |
|-----------------|----|-----------------|-----------------|
| | | Mean ± SE | Mean ± SE |
| Organic 2010 | 30 | 0.24 ± 0.01 (b) | 0.70 ± 0.01 (c) |
| Biodynamic 2010 | 30 | 0.19 ± 0.01 (c) | 0.64 ± 0.01 (d) |
| Organic 2011 | 30 | 0.30 ± 0.01 (a) | 0.76 ± 0.01 (a) |
| Biodynamic 2011 | 30 | 0.26 ± 0.01 (b) | 0.73 ± 0.01 (b) |

^a Legend: N = number of samples, SE = standard error; different letters indicate significance at $p \leq 0.05$.

(*i.e.* forms were rounder and more solid) than for the biodynamic samples in both harvesting years (Table 2). Similarly the value of film intensity was significantly different for organic and biodynamic samples in both years, but no uniform trend could be observed (Table 1). On the other hand, count and total area significantly differentiated the wines from the two cultivation systems in 2011 only (Table 1).

3.3. Chemical analyses of wine samples

The contents of ethanol, dry matter, reducing sugar, total phenolics and the red colored compounds showed high values in harvesting year 2011, and the main parameters that showed a significant difference between the two cultivation systems were the total phenolics and the red colored compounds (OD 520 nm) (Table 3). It is well known that the color of red wines is due to the presence of phenolic antioxidant compounds, *i.e.* anthocyanins. Although red grapes from biodynamic management were found to be more rich in anthocyanins compared to

grapes from organic viticulture,⁷ previous study has shown that highly colored grapes do not necessarily produce highly colored wines, being any difference probably related with the easiness of anthocyanins to be extracted from grape skins into musts.²⁰

Based on the image analysis of the residue morphology of red wine droplets the hypothesis that the wine DEM structure are mainly composed of potassium bitartrate (KHT) crystals is formulated. Previous findings confirm that the crystalline deposits in wine are mainly caused by an excess of KHT, the crystallization of which is inhibited by wine phenolics, due to their adsorption on the crystal sides, resulting in significant changes in the crystal shape and size, consequently crystals appeared to be thin plates.²¹

3.4. Relationship between DEM structures and wine composition

As reported in Table 4, the sizing-parameters regarding the crystalline structures were negatively correlated with the wine composition, whereas the film intensity exhibited a positive correlation. This newly discovered negative correlation between the chemical composition and the total area covered by structures (needle-like and flower-like) can be explained by the two-phase evaporation process observed in wine droplets and already reported for blood droplets.²² Moreover, the regressive correlation between the film intensity and the total area ($r = -0.73^*$; $p < 0.05$) indicates that the thicker is the film the smaller is the total area of the emerging structures.

As far as shape-descriptors are concerned (*i.e.* circularity and solidity), positive values of significant correlations with the wine composition were always obtained (Table 4), and the highest values were observed for red colored compounds (OD 520 nm)

Table 3 Statistics of the Sangiovese red wine composition for the organic and biodynamic samples per harvesting year^a

| Wine trial | N | Alcohol (% v/v) | Sugar (g l ⁻¹) | Dry matter (g l ⁻¹) | OD 520 nm (AU) | pH | Total phenolics (mg l ⁻¹) |
|-----------------|---|-----------------|----------------------------|---------------------------------|----------------|-----------------|---------------------------------------|
| | | Mean ± SE | Mean ± SE | Mean ± SE | Mean ± SE | Mean ± SE | Mean ± SE |
| Organic 2010 | 2 | 12.0 ± 0.3 (ab) | 1.19 ± 0.19 (a) | 22.8 ± 0.6 (ab) | 2.9 ± 0.1 (b) | 3.54 ± 0.06 (a) | 1157 ± 66 (b) |
| Biodynamic 2010 | 2 | 11.1 ± 0.3 (b) | 1.01 ± 0.01 (a) | 20.2 ± 0.7 (b) | 2.1 ± 0.3 (c) | 3.56 ± 0.01 (a) | 961 ± 55 (c) |
| Organic 2011 | 2 | 14.0 ± 0.7 (a) | 1.49 ± 0.21 (a) | 25.8 ± 1.5 (a) | 4.5 ± 0.1 (a) | 3.43 ± 0.00 (b) | 1632 ± 177 (a) |
| Biodynamic 2011 | 2 | 13.1 ± 1.0 (ab) | 1.45 ± 0.15 (a) | 25.0 ± 1.3 (a) | 3.7 ± 0.2 (a) | 3.55 ± 0.02 (a) | 1520 ± 38 (a) |

^a Legend: N = number of samples; SE = standard error; different letters indicate significance at $p \leq 0.05$.

Table 4 Correlations (*r*) between the DEM data (sizing-parameters, shape-descriptors) and the wine chemical parameters^a

| Wine parameter | Count | Total area | Structure area | Perimeter | Film intensity | Circularity | Solidity |
|---------------------------------------|----------|------------|----------------|-----------|----------------|-------------|----------|
| Alcohol% (v/v) | -0.43 ns | -0.68 ns | -0.53 ns | -0.71* | 0.77* | 0.72* | 0.76* |
| Sugar (g l ⁻¹) | -0.54 ns | -0.79* | -0.53 ns | -0.77* | 0.77* | 0.75* | 0.73* |
| Dry matter (g l ⁻¹) | -0.41 ns | -0.72* | -0.65 ns | -0.82* | 0.70* | 0.81* | 0.86** |
| OD 520 nm | -0.13 ns | -0.50 ns | -0.67 ns | -0.83* | 0.56 ns | 0.90** | 0.94*** |
| pH | -0.49 ns | 0.41 ns | -0.33 ns | 0.41 ns | 0.26 ns | -0.42 ns | -0.09 ns |
| Total phenolics (mg l ⁻¹) | -0.35 ns | -0.77* | 0.01 ns | -0.88** | 0.72** | 0.91** | 0.56 ns |

^a Legend: ns = $p > 0.05$; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

and total phenolics, therefore suggesting that anthocyanins seems to affect the shape of crystals to a large extent.

4. Conclusions

The results of this preliminary study suggested that DEM structures were affected by the chemical composition of red wine, in particular the phenolic compounds, and the crystallographic approach was able to discriminate the wines depending on cultivation systems, *i.e.* organic *vs.* biodynamic, when considering the shape-descriptors. This result is of particular interest due to the current lack of analytical methods able to disclose the origin of wines from biodynamic agricultural management.

Acknowledgements

The authors thank Antonello Russo (Demeter Italy) for funding this research, and Edda Sanesi, and Mario Dorigato for their support.

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