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Volatile fraction analysis by HS-SPME/GC-MS and chemometric modeling for traceability of apples cultivated in the Northeast Italy

Vanessa Giannetti [a,*], Maurizio Boccacci Mariani [a], Paola Mannino [a], Federico Marini [b]

[a] Department of Management, Sapienza University of Rome, Via del Castro Laurenziano 9, 00161 Rome, Italy
[b] Department of Chemistry, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy

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ABSTRACT

The present study aimed at characterising the flavour composition of apple cultivars grown in the Northeast Italy through different cultivation methods, by combining Head Space-Solid Phase Micro Extraction/Gas Chromatography Mass Spectrometry (HS-SPME/GC-MS) analysis of volatile fraction with chemometric tools for class modeling. In order to represent the overall production in the target area, the investigation included 42 apples varieties consisting of ancient, non-native and new hybrid cultivars grown in Friuli Venezia Giulia and Alto Adige-South Tyrol, respectively. Moreover, apple samples from both conventional and organic agricultural practices were considered. Overall 118 volatile compounds were identified in the samples and Partial Least Squares-Discriminant Analysis (PLS-DA) was used to classify apples based on their different geographical origin or growing conditions. Models highlighted good classification results both in calibration (over 91%) and cross-validation (over 87%), enabling to obtain a good separation between apple categories with high prediction accuracy (over 90%). In addition, the Variable Importance in Projection (VIP) scores of the PLS-DA models were calculated, allowing to identify a reduced number of volatiles (e.g., ethanol, ethyl acetate, isobutyl acetate, propyl propanoate, 1-hexanol, D-limonene, (Z)-2-hexen-1-ol acetate and others) which are relevant for the discrimination of different apple groups. The proposed approach may represent a powerful tool for fruit traceability.

1. Introduction

About 7.500 varieties of apples (*Malus domestica Borkh*) are grown all over the world. Italy is the fifth country for annual production (2.460.000 tons in 2014) and the third, after China and Poland, for export of apple fruits (about 30% of total production) (World Apple and Pear Association (WAPA), 2014). Over 80% of the national crop comes from the Northeast Italy: the region of Trentino Alto Adige represents the main producer (59.3%), followed by Veneto (13.8%), Emilia Romagna (9.6%) and Friuli Venezia Giulia (1.9%) (Dal Piaz, 2015). Market apples production essentially relies on few extensively-cultivated varieties introduced in Italy less than 25 years (mainly *Golden, Gala, Jona, Fuji*). However, there is also a growing interest in the recovery of ancient cultivars as a part of experimental projects for the protection of endangered species (ISPRA, 2014) and the establishment of breeding programs aimed at producing new apples varieties, more resistant to disease and/or with peculiar sensory properties (Dunemann, Ulrich, Boudichevskaia, Grafe, & Weber, 2009; Hampson et al., 2000; Rowan, Hunt, Alspach, Whitworth, & Oraguzie, 2009). Indeed, among the organoleptic characteristics that most affect consumer preferences, flavour appear to be crucial in the differentiation of apple cultivars (Corrigan et al., 1997; Hampson et al., 2000; Mehinagic et al., 2003). For this reason, the biogenesis of apple odorants have been extensively studied in the past and over 350 volatiles belonging to the classes of esters, alcohols, aldehydes, acids, ketons, norisoprenoids and terpenoids have been identified (Maarse, 1991; Yahia, 1994). In particular, screening of volatile metabolites has been used to characterize some intensively cultivated apple varieties (*Royal Gala, Fuji, Pink Lady*) in relation to their genotype (Aprea et al., 2012; Dunemann et al., 2009; Schaffer et al., 2007) and enzymes pattern (Echeverria et al. 2004a; Villatoro et al., 2008). However, development and composition of apple flavour is greatly influenced also by on-tree ripening conditions (e.g., territory of cultivation, seasonal variation, maturity stage at harvest) (Echeverria et al. 2004b; Mehinagic, Royer, Symoneaux, Jourjon, & Prost, 2006; Villatoro et al., 2008; Watada & Abbott, 1985) and post-harvest processing factors (e.g., storage technologies, temperature

* Corresponding author.
E-mail address: vanessa.giannetti@uniroma1.it (V. Giannetti).

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and duration) (Echeverria et al. 2004b; Dixon & Hewett, 2000; Lara et al., 2006; Lopez et al., 2000). In addition, many efforts have been devoted in the last decade to the interpretation of chemical data available on apples' nutritional and sensory properties in relation to different production systems (e.g. integrated and organic farming) (Hecke et al., 2006; Peck, Andrews, Reganold, & Fellman, 2006; Roth et al., 2007; Valavanidis, Vlachogianni, Psomas, Zovoili, & Siatis, 2009). Accordingly, the study of apples' volatiles by combining chemical analysis with chemometric tools could offer a powerful approach to trace each fruit "*from farm to fork*", providing fingerprint information on its geographical origin, period of harvest, method of cultivation, storage conditions and shelf-life for fresh consumption.

To the best of the authors' knowledge, no comprehensive data have been reported so far on the volatiles composition of different apple varieties cultivated in the Northeast Italy. The present research aims at characterizing the flavour profile of apple cultivars grown in Alto Adige-South Tyrol (AA-ST) and Friuli Venezia Giulia (FVG), respectively. Currently, several non-native cultivars are widely grown in the target territory (e.g. *Golden Delicious* covers more than 40% of total production) and there is also a relevant interest in the cultivation of new hybrids (e.g., *Pink Lady, Florina, Delorina, Topaz*) (ISPRA, 2014; Tagliavini, 2014). AA-ST contributes to 25% of apples organic production in EU (FAO, 2014) and about 30 apple varieties (mainly *Royal Gala, Braeburn, Golden Delicious, Pink Lady*) are currently obtained from both organic and conventional farming. With the exception of *Renetta* (about 20% of total apple production), cultivation of ancient cultivars in Northeast Italy is limited to small areas within the framework of research programmes aimed at integrating native varieties in the local market (Dal Piaz, 2015; ISPRA, 2014). The present study investigated 42 apple cultivars including native, non-native and hybrid varieties in order to represent overall production in the considered area. Attention focused on 14 ancient cultivars grown under organic farming in the pre-Alpine area of Pordenone province (FVG) as a part of the "*Ancient apples project*". The main goals of the project are to undertake a census of ancient cultivars still grown in their native area in order to ensure biodiversity conservation, as well as to promote reintegration of these apples into the market.

In this work, apple samples were grouped taking into account their geographical origin (AA-ST or FVG) and growing conditions (organic or conventional fruit cultivation) in order to study different possibly discriminating factors through multivariate statistical analysis of flavour data obtained by the Headspace Solid-Phase Micro Extraction technique (HS-SPME) coupled to gas-chromatography mass spectrometry (GC-MS). The identification of a peculiar mixture of odorants may represent a key strategy for the traceability of apples grown in different Italian regions, as well as for assessing genuinity of organically grown apple fruits compared to conventionally cultivated ones.

2. Materials and methods

2.1. Sample collection and preparation

Samples used for this study belonged to 42 different apple varieties. The first group included 22 ancient cultivars (*Belladonna, Bislung dal bosc, Calvilla, Clavilla Bianca, chei da la Rosa, Cigulin, Coston, da la Fraula, dal Ciopeit, dal Cordon, di Corone, dal Dolc, dal Poç, di Giuliu, Limoncella, Marc Panara, Non Id, Pomacis, Rosso Invernale, Ruggine Gialla, Striato Dolce, Zeuka*), which were organically grown in the area of Pordenone province within the frame of the "*Ancient apples project*" from 2014 to 2015 harvests. The remaining samples were apples from Val Venosta in Bolzano province (AA-ST): a group included 7 non-native cultivars (*Braeburn, Fuji, Golden Delicious, Granny Smith, Pinova, Jona Red, Stark Delicious*) and 4 new hybrid scab-resistant apple varieties (*Delorina, Gold Rush, Rubens, Topaz*), all grown under organic agricultural practices in 2014. The other group comprised 1 native cultivar (*Renetta del Canada*) and 8 non-native varieties (*Fuji, Golden Delicious, Granny Smith, Idared, Modi, Morgenduft, Pink Lady, Red Delicious*), grown following conventional agriculture system in 2014. In all cases, samples were analyzed relatively shortly after harvest, so to avoid any possible effect of prolonged storage on the stability of their volatile profile.

Sample preparation was performed according to the method of Aprea et al., 2011, slightly modified as follows. Twenty grams of each apple sample (pulp) were cut into small pieces, inserted in a glass vessel and mixed with 50 mL of HPLC grade water (Milli-Q system by Millipore, Bedford, MA, USA) and 10 g of sodium chloride (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) with the aid of a domestic grinder (Ariete 510 model). Sodium chloride was added to inhibit enzymatic reactions and also to promote extraction efficiency of volatile compounds in the head space due to salting out effect. A 10 g aliquot of homogenized sample was transferred into a 20 mL vial, then sealed with PTFE/silicone septum and aluminum crimp cap (Macherey-Nagel, Bethlehem, PA, USA).

2.2. HS-SPME/GC-MS analysis

Headspace SPME and GC-MS analysis were performed according to the method of Dunemann et al., 2009. HS-SPME procedure was carried out using a 50/30 μm (1 cm) divinylbenzene/carboxen/polydimethylsiloxane fiber (Supelco, Bellefonte, PA, USA) with the aid of a TriPlus RSH autosampler (Thermo Fisher Scientific, Whaltam, MA, USA). The GC-MS system consisted of a Trace GC-Ultra gas chromatograph equipped with a Programmed Temperature Vaporized (PTV) injector (glass liner, 2 mm i.d., 2.75°.d., 120 mm) and interfaced with a single quadrupole ISQ LT mass spectrometry (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was performed by a polyethylene glycol (PEG) capillary column (HP-INNOWAX, 30 m, 0.25 mm i.d., 0.15 μm) purchased by Agilent Technologies (Santa Clara, CA, USA) and operating with helium as carrier gas at flow rate of 0.8 mL/min. The fiber was preconditioned before the analyses according to manufacturer's instructions, performing two blank injections at temperature of 270 °C. For analysis, the extraction temperature was kept at 40 °C to avoid degradation of thermally unstable compounds in apple pulp. Samples were incubated for 10 min and then the fiber was exposed for 15 min in the head space. During incubation and extraction steps the sample was kept under agitation. After extraction, volatile compounds were thermally desorbed for 3 min in the GC injector port held at 230 °C. The injection was performed in splitless mode (splitless time: 1 min), then a split ratio of 10 was setted. The GC oven temperature was programmed at 40 °C for 3 min, then ramped at 8 °C/min up to 220 °C (analysis time: 26 min). The mass spectrometry operated in Electron Impact mode at 70 eV and in scan range (m/z) from 40 to 350 a.m.u., with both ion source and transfer line at 250 °C. The identification of volatile compounds was carried out by comparison of their mass spectra with those of the National Institute of Standards and Technology (NIST) mass spectral database. According to accepted rules defined by the Metabolomics Standards Initiative (Sumner et al., 2006), qualitative analysis based upon spectral similarity with commercial spectral library, without use of chemical reference standards, should be reported as "putative compounds identifications" or "level 2 identifications". As spectral evidence to validate the metabolite identifications, both Kovats Retention Indices (RI) and Reverse Search Indices (R match) were estimated. RI were calculated using *n*-alkanes (C_8–C_{20}) as external references and compared with literature data. However,