Ancient apple cultivars are known for their organoleptic properties over a small geographic area, but little is known of their nutraceutical properties which might be useful in large-scale breeding programs. Nine ancient apple cultivars from Tuscany (Italy) were characterized for their organoleptic properties, phenolic profile and antioxidant activity. These cultivars had a high polyphenol concentration (principally flavanols and phenolic acids) and high total antioxidant capacity compared to most commercial apple cultivars. Fruit from the cultivars 'San Michele' and 'Del Debbio' showed a good compromise between fruit size and solid soluble content and might be suitable for fresh consumption, while fruit from 'Benito', 'Della Piastra', 'Lugliese Grisanti', 'Del Sangue' and 'Ruggine' had a high polyphenol content and excellent antioxidant capacity and may be suitable for breeding programs. 'Ruggine' fruit could also be used for sweet juices with high nutraceutical properties due to a high soluble solid content and high flavanoid concentration.
Pisa, 15th January 2019

The Editor of *Food Chemistry*

Dear Editor,

We have revised our manuscript: “Ancient apple cultivars from Garfagnana (Tuscany, Italy): a potential source for ‘nutrafruit’ production” following the suggestions of the referee 1 and receiving editor. We are grateful to both for their useful comments which have been very helpful in improving the manuscript. Please see in the attached file “responses to referees’ comments” details of how we have accommodated the referees’ suggestions and comments.

Best Regards,

Dr. Marco LANDI, PhD

Department of Agriculture, Food & Environment
University of Pisa
Via del Borghetto, 80 - 56124, Pisa, Italy
Email: marco.landi@agr.unipi.it
Tel: +39 0502216620
The Editor of Food Chemistry

Dear Editor,

We have revised our manuscript: “Ancient apple cultivars from Garfagnana (Tuscany, Italy): a potential source for ‘nutrafruit’ production” following the suggestions of the referee 1 and receiving editor. We are grateful to both for their useful comments which have been very helpful in improving the manuscript. Please see below details of how we have accommodated their suggestions and comments.

COMMENTS

Reviewer 1: It is appropriate to give brief information about the general characteristics, consumption patterns and production quantities of these local apple varieties in the introduction section of the article.

Our response: we already considered these aspects of crucial importance when writing the manuscript; however, our local varieties belong to a local germplasm collection and the data required by the referee are not available.

In the conclusion section, the findings are written as a result. Overall contribution to the science of this study should be written in more detail to the conclusion section.

Our response: thank you for that suggestion. We completely agreed with the referee’s comment and therefore the conclusion section was completely rewritten with the goal to highlight the contribute to the science of our study rather than simply summarizing the main findings of our research.

The highlights are not remarkable. They should be reviewed.

Our response: We have completely refresh the highlights with the same attempt (of the previous point) to figure out the main contributes to the science of our study as well as its core of novelty.
This research is important for the introduction and dissemination of local apple varieties in Italy. However, the research seems to be more important on a local scale. So, more detailed information about the overall contribution of this research should be given.

Our response: Thank you for this critic point of view about this point of weakness. In accordance, we have mentioned in key sections (abstract, discussion, conclusions) that the use different old varieties, as those screened in our study, can be relevant not only at a local scale. We highlighted that some cultivars could be useful for different purposes on the base of their different characteristic; in particular, some of them could be useful for large-scale breeding programs, other cultivars for the production of juice with a high nutraceutical values, which is not only restricted to local uses.

Line 108-109 and Line 270-273 are written red underlined.
Our response: we apologize for this. The sentences were corrected.

Our response: the reference was deleted from the references’ list.

RECEIVING EDITOR'S COMMENTS:

The highlights are intended to showcase your most important results. Currently, they are mundane and lack context, requiring that the manuscript is read first. REVISE.

Our response: We have completely refresh the highlight with the attempt to figure out the main contributes to the science of our study as well as its core of novelty.

20 In many countries where apples have been growing for centuries, ancient apple cultivars are known locally, but few data are available describing the potential benefits of consumption or features that could be useful for apple breeding programs. Nine ancient cultivars from Garfagnana (Tuscany, Italy) were characterized for their phenolic profile and antioxidant activity. Besides cultivar-specific features, generally, a high polyphenol content (principally flavanols and phenolic acids) and total antioxidant activity were found with respect to these ancient apple cultivars. 'Rossa di Corfino' and 'Del Giappone' did not present relevant quality characteristics.
Our response: we have considered the changes proposed by the referee when revising the manuscript. However, the final outcome can be slightly different given that the final version was revised by a native speaker who also simplify and merged some sentences together.

27 Conversely, 'San Michele' and 'Del Debbio' were suitable for fresh consumption. - presumably most eating apples are "suitable for fresh consumption", be more specific.
Our response: Thank you for this suggestion. We have refreshed as follow: “‘San Michele’ and ‘Del Debbio’ fruits had showed a good compromise between fruit size and solid soluble content and might were suitable for the fresh consumption.”

27 ‘Benito’, ‘Della 27 Piastra’, ‘ Lugliese Grisanti’, ‘Del Sangue’ and ‘Ruggine’ had a high polyphenols content and excellent antioxidant activity, making them also suitable for apple breeding programs. Finally, ‘Ruggine’, due to high soluble solid content and flavanol content, could be used for production of juice with putative health benefits.

Our response: we have considered the changes proposed by the referee when revising the manuscript. However, the final outcome can be slightly different given that the final version was revised by a native speaker who also simplify and merged some sentences together.

35 Apples cultivation in Western countries offers a high return on investment, largely because of mechanization, although highly skilled labour is need. Although hundreds of apple cultivars exist, globally, production is linked to just a few (…), such as Red Delicious, Golden Delicious, Gala and Fuji. The selection of these popular and extensively utilised cultivars has results in a uniformity of commercial apple orchards (…) and a dramatic loss in genetic biodiversity. However, interest in the preservation of autochthonous genetic heritage of fruit species is growing (…): ancient autochthonous varieties could represent an important source of innovative characteristics to satisfy modern fruit culture, such as shape and colour. However, the size, taste and texture of ancient cultivars is not always commercially attractive, e.g. leathery peel, astringent taste, and a small size.

Our response: we have thoroughly considered the changes proposed by the referee when revising the manuscript. However, the final outcome can be slightly different given that the final version was revised by a native speaker who also simplify and merged some sentences together.

76 Standards for polyphenol profile and 2,2-diphenyl-1-picrylhydrazil (DPPH) were purchased from Sigma-Aldrich (Milan, Italy).

Our response: done

85 About 3 kg of fruits from each cultivar were picked randomly on the commercial ripening date from specific positions on the trees, so that the micro-meteorological and edaphic impact was comparable.

Our response: Thank you for this comment. We have revised as follow: “About 3 kg of fruit (from three different trees per cultivar) were randomly picked from each cultivar at a consistent position from the tree canopy at the commercial maturity date, so that the micro-meteorological and environmental impacts were comparable.”

149 Organoleptic properties of the nine ancient apple cultivars are summarized in Table 1. It is a very basic requirement in scientific writing that actions are reported in the past tense (work was done) and results in the present tense (results are shown)

Our response: Thank you for this comment. We corrected the sentences in which the verb tense were wrong.
There are significant issues with the English including syntax, grammar, tense and choice of words. These must be resolved with the help of a native speaker or English language editing service, such as that provided by Elsevier https://webshop.elsevier.com/languageservices/languageediting. The authors are advised strongly again trying to make linguistic corrections independently, as this will further delay the paper and may lead to it being rejected.

**Our response:** the manuscript was carefully revised by a native speaker who is Dr. Annette Richardson from The New Zealand Institute for Plant & Food Research Limited (NZ). Meanwhile editing the manuscript, Dr. Richardson also raised some comments and proposed some suggestions to be undertaken. Therefore, all the co-authors are thankful to Dr. Richardson who was mentioned in “Acknowledgements”.

We hope that in this revised version the manuscript might be appropriate for the final acceptance.

Best Regards,

Dr. Marco LANDI, PhD

Department of Agriculture, Food & Environment
University of Pisa
Via del Borghetto, 80 - 56124, Pisa, Italy
Email: marco.landi@agr.unipi.it
Tel: +39 0502216620
Highlights

- Nine ancient Tuscan apple cultivars were analysed for their nutraceutical properties
- Organoleptic characteristics and phenol profile varied among cultivars
- UPLC–MS coupled with PCA is a useful tool for apple cultivar screening
- Different possible uses are proposed for each cultivar

- Polyphenolic profile was determined by UPLC-MS in ancient Tuscany apple cultivars
- Flavanols and phenolic acids are the most abundant polyphenols in ancient apples
- Procyanidin B1 has the highest correlation with the fruit’s antioxidant capacity
- Phenolic content and antioxidant ability are higher than most cultivated apples
- Ancient apples should be re-evaluated for a “nutrafruit” production
Ancient apple cultivars from Garfagnana (Tuscany, Italy): a potential source for ‘nutrafruit’ production

Ermes Lo Piccolo, Marco Landi, Rossano Massai, Damiano Remorini, Giuseppe Conte, Lucia Guidi

a Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto, 80 - 56124 Pisa (Italy)
b Interdepartmental Research Center Nutrafood "Nutraceuticals and Food for Health", University of Pisa, Via del Borghetto, 80 – 56124 Pisa (Italy)

* Corresponding author:
Dr. Marco LANDI
Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto, 80 - 56124 Pisa (Italy)
Tel. +39 50 2216620
Email: marco.landi@agr.unipi.it

Abstract

In many countries where apple has been growing since centuries, ancient apple cultivars are known for their organoleptic properties over a small geographic area the local level, but few data are available little is known of their nutraceutical properties, which features that might could be useful for large-scale genetic breeding programs. Nine ancient apple cultivars from Garfagnana (Tuscany, Italy) were characterized for their organoleptic qualities, phenolic profile and antioxidant activity. Besides cultivar-specific features, these cultivars had generally a high polyphenol concentration (principally flavonols and phenolic acids) and high total...
antioxidant capacity were found with respect compared to most commercial apple cultivars. Cultivars ‘Rossa di Corfino’ and ‘Del Giappone’ did not show relevant quality characteristics. Fruit from the cultivars – Conversely, ‘San Michele’ and ‘Del Debbio’ fruits had showed a good compromise between fruit size and solid soluble content and might be suitable for the fresh consumption, while fruit from ‘Benito’, ‘Della Piastra’, ‘Lugliese Grisanti’, ‘Del Sangue’ and ‘Ruggine’ showed a high polyphenol content and excellent antioxidant capacity making them and may be also suitable for breeding programs. Finally, ‘Ruggine’ fruits could also be used for the sweet juices with high nutraceutical properties because of due to high soluble solid content and high flavanol amount concentration can be used for production of juice with high nutraceutical properties.

Keywords: ancient cultivars, antioxidant capacity, apple, cluster analysis, organoleptic properties, phenolic profile
1. Introduction

Apple cultivation in Western countries offers a high return on investment, largely because it represents one of the high-income crops, with a high degree of mechanization, level and although highly skilled labour is also required. Although hundreds of apple cultivars exist globally, most apple due to a large number of breeding programs, in the world the production is linked to just a few groups of cultivars (Hokanson, Lamboy, Szewc-McFadden & McFerson, 2001) such as ‘Red Delicious’, ‘Golden Delicious’, ‘Gala’ and ‘Fuji’ (Hokanson, Lamboy, Szewc-McFadden & McFerson, 2001). In addition, the clonal selection of these popular and extensively utilised cultivars has resulted in a uniformity of commercial apple orchards (Donno et al., 2012; Cerrutti, Bruun, Donno, Beccaro & Bounous, 2013); and which has led to a dramatic loss in genetic biodiversity.

Nowadays, the interest for the preservation of autochthonous genetic heritages of fruit species is growing (Wojdylo, Oszmiński & Laskowski, 2008; Cerrutti et al., 2013; Ferreira et al., 2016). The ancient autochthonous varieties could represent an important source of innovative characteristics to satisfy the needed for modern fruit-culture, such as particular fruit shapes, new tastes and different peel and pulp colours in peel and pulp. However, often fruit of these ‘ancient’ cultivars also display unappreciated, unacceptable organoleptic qualities such as leathery peel, astringent taste compounds and but also a small size making them commercially unattractive.

In the last years, the concept of food in Western countries has changed in Western countries whereas, in addition to excellent organoleptic characteristics, consumers also require food with nutraceutical properties useful for that benefit human health (Lobo, Patil, Phatak & Chandra, 2010; Roche et al., 2015). Surely, undoubtedly, one of the most important features that make apple fruits interesting for researchers is their high polyphenolic content in polyphenols (Jakobek & Barron, 2016; Jakobek, García-Villalba & Tomás-Barberán, 2013; Maragò, Michelozzi, Calamai, Camangi & Sebastiani; 2016). These metabolites have been intensively studied in epidemiological research because they are particularly important for the human diet as they...
play a positive role in reducing the risk of many cardiovascular diseases and showed anticancerogenic properties (Rasouli, Farzaei & Khodarahmi, 2017). The main polyphenols contained in apple fruits are anthocyanins, dihydrochalcones, flavanols, flavonols, hydroxybenzoic acid and hydroxycinnamic acids (Neveu et al., 2010). However even though their the concentration of polyphenols in fruit is can be strongly influenced by many factors such as including geographical region, agronomic techniques, conservation—fruit storage technique method, stage of fruit maturity and as well as fruit variety—cultivar (Awad, Wagenmakers & De Jager, 2001; Treutter, 2001).

Besides of constitutive high level of phenols in apple cultivars, it has been displayed in many cases suggested that ancient apple cultivars could have a higher content of these—nutraceutical compounds compared to than commercial apple cultivars (Maragò et al., 2016; Iacopini, Camangi, Stefani & Sebastiani, 2010). Therefore, the aim—goal of new researches should be the selection of some ancient cultivars with appreciable organoleptic characteristics and nutraceutical content for the fresh fruit market or, alternatively, the selection of ancient cultivars more with suitable properties for juice production.

In this work, we characterized the fruit of nine ancient Italian apple cultivars from Italy (Garfagnana, Tuscany) for their organoleptic qualities, polyphenols profile and antioxidant activity, and on the basis of used these ir-characteristics we to suggest different—possible utilization uses for cultivars. The cultivars are enrolled in the bank of germplasm of Tuscany region (Regional Law N. 64, 16th November 2004).

2. Materials and methods

2.1. Reagents, solvents and standards

Methanol, formic acid and acetronile were purchased by from Carlo Erba Reagents (Cornaredo, Milan, Italy). Standards for polyphenol profile and 2,2-diphenyl-1-picrylazil (DPPH) were purchased by from Sigma-Aldrich (Milan, Italy).
2.2. Apple sampling and organoleptic characteristics

Fruits from ancient apple cultivars (*Malus domestica* Borkh.; 3-5 plants per cultivars) were harvested from an orchard situated at the experimental field “Centro vivaistico La Piana” in Camporgiano (44°08’17’’ N, 10°19’05’’ E; *Lucca, Italy*), which is located in the historical and geographical region of Garfagnana, *Lucca* (Italy). The nine cultivars have been identified by the abbreviations: B (‘Benito’), RC (‘Rossa di Corfino’), DP (‘Della Piastra’), DG (‘Del Giappone’), LG (‘Lugliese Grisanti’), SM (‘San Michele’), DD (‘Del Debbio’), DS (‘Del Sangue’), R (‘Ruggine’).

About 3 kg of fruit (from three different trees per cultivar) were randomly picked of fruits from each cultivar were picked randomly (but at a consistent distance from the tree canopy) at the commercial ripening-maturity date randomly from specific positions on the trees, so that the micro-meteorological and edaphic-environmental impacts were comparable. Morphometric analyses of fruit were carried out using standard descriptors for apples (Bellini, Giordani, Picardi & Giannelli, 2008), using ten fruits of each cultivar. Physical and chemical harvest indexes were estimated on three randomly selected fruits of each cultivar as follows: (i) fresh weight (FW) (g), height (mm), width (mm) and height/width ratio of fruits; (ii) flesh firmness (expressed as Kg cm$^{-2}$) was measured on a sample of fruits after removing the skin, on two opposite sides of each fruit, with a digital penetrometer equipped with an 11 mm tip (TR Snc, Forlì, Italy); (iii) soluble solid content (SSC, °Brix) was determined on a flesh juice sample using a digital refractometer (refractometer Mod. 53 011, Turoni, Forli, Italy). Harvested fruits were then stored in a cold chamber at 2 °C and 95% relative humidity for one month before biosampling chemical analysis. At sampling, fruits were carefully washed twice with tap water and finally once with distilled water. Fruits were then peeled and sliced with a sharp knife before removing the core portion and seeds. After that, the slices were then...
cut into small portions (approximately 10 x 10 x 50.5 mm), randomly allocated to falcon tubes, frozen in liquid nitrogen and stored at -80 °C until for biochemical analyses.

2.3. Phenol extraction

Flesh samples (about 1 g FW) were homogenized with 10 mL of 70% (v/v) 99.5% HPLC grade methanol by sonication for 30 min, keeping the temperature from 0 to 4 °C. After centrifuging samples at 6000 g for 10 min at 4 °C, the supernatant was collected and filtered with PTFE filters (0.20 μm pore size; Sarstedt, Verona, Italy). Each sample replicate consisted of FW material belonging to three different fruits of the same tree. Extracts were stored at −80 °C before analysis.

2.4. UPLC–MS analysis method

The phenolic profile was determined as described in Assumpção et al. (2018) with few modifications. The UPLC–MS analysis was performed using an Agilent 1290 Infinity II LC system (Agilent Technologies Italia S.p.A., Cernusco Sul Naviglio, Italy) consisting of a degasser, a binary pump, an autosampler, a column oven and equipped with an Agilent 6495A triple quadrupole MS with a C18 column, 2.1 × 50 mm, 1.8 μm (Agilent Zorbax Eclipse Plus, Santa Clara, CA, USA) was used for separation of phenolic compounds. Solvent A consisted of 0.2% formic acid in water whereas solvent B was 0.2% formic acid in acetonitrile. The elution gradient was: 6% B (3 min), from 6 to 30% B (in 11 min), from 30 to 100% B (in 2 min), 100% B (2 min). The column temperature was 35 °C, the flow rate was 0.3 mL min⁻¹, and the injection volume was 2x10⁻⁶ L. MS parameters employed were as follows in ESI(+) mode: gas temp: 150 °C; gas flow: 13 L min⁻¹; nebulizer: 50 psi; sheath gas heater: 350 °C; sheath gas flow: 12 L min⁻¹; capillary: 3500 V; HPRF funnel: 120; LPRF funnel: 40; in ESI(−) mode: gas temp: 150 °C; gas flow: 13 L min⁻¹; nebulizer: 50 psi; sheath gas heater: 350 °C; sheath gas flow: 12 L min⁻¹; capillary: 1500 V; HPRF funnel: 120; LPRF funnel: 80. For quantification, an external standard method was used. A calibration curve was...
constructed for each compound analysed with at least five different concentrations from 1 to 500 μg L⁻¹ was constructed for each compound analysed and utilized to quantify the concentration of each compound in the samples. Data are expressed as μg g⁻¹ FW.

### 2.5. Antioxidant capacity activity

To assay the antioxidant capacity, was measured following the method reported by Brand-Williams, Cuvelier and Berset (1995) was followed. Briefly, 15 μL of phenolic extract were added to 990 μL of a solution containing 3.12x10⁻⁵ M DPPH in methanol. The decrease in absorbance at 515 nm was measured against a solution blank (without extract) after a reaction time of 30 min at room temperature (that was preliminary optimised to observe for the highest antioxidant effect of concentrations in the extract) using a spectrophotometer (Ultrospec 2100 pro, GE Healthcare Ltd., Chalfont St. Giles, Buckinghamshire, UK). Results were expressed as percentage of reduction of the initial DPPH absorption by the extracts and expressed as μmol Trolox g⁻¹ FW.

### 2.6. Statistical analysis

Data are expressed as mean ± standard deviation and were subjected to a one-way ANOVA test and statistical differences among the nine apple cultivars were calculated by least significant difference (LSD) test at 95% of confidence with GraphPad Software (GraphPad, La Jolla, USA). Data are expressed as mean ± standard deviation. Pearson’s correlation coefficient between phenolic compounds and total antioxidant activity was carried out with GraphPad software. Hierarchical clusters to group cultivars with similar phenolic profiles, were determined using Ward’s method, in order to see similarities between cultivars and which phenols most contributed to creating

...
distinctions\ differs\ in\ the\ dataset.\ The\ Cluster\ and\ PCAs\ analysis\ were\ conducted\ using\ IBM\ SPSS\ Statistics\ 24\ (IBM,\ New\ York,\ USA).

3. Results and Discussion

3.1. Organoleptic characteristics

Organoleptic properties of the nine ancient apple cultivars were summarized in Table 1. Significant differences were revealed among the apple cultivars for fruit weight, height, width, firmness and soluble solids content (SSC). Fruit fresh weight varied from 88.8 g for LG to 256.0 g for SM having the highest weight and LG the lowest one (Table 1). The cultivar SM together with the cultivar DD also had the highest fruit height and width and—In addition, DD fruits\ also had\ the\ highest\ height/width\ ratio\ (1.02 \pm 0.02)\ as\ compared\ to\ while\ fruit\ from\ the\ average\ all\ the\ other\ cultivar\ fruits\ (values\ ranged\ from\ 0.83\ to\ 0.86; mean average 0.84 \pm 0.04).

Lower values of fruit flesh firmness were recorded in the cultivars LG, DS and DD cultivars (4.38, 6.86 and 6.94 kg cm$^{-2}$, respectively) and the highest values were in fruit from B and DP cultivars (9.97 and 9.43 kg cm$^{-2}$, respectively). In most cases the values of fruit flesh firmness from this study were similar to those found in commercial cultivars harvested at commercial maturity—as in this study (Iacopini et al., 2010). Lower values were recorded in LG, DS and DD cultivars (4.38, 6.86 and 6.94 kg cm$^{-2}$, respectively) and the highest in B and DP cultivars (9.97 and 9.43 kg cm$^{-2}$, respectively).

The soluble solid content of fruit is an important quality parameter to test mainly indicator particularly of the sugar content in apples. For values of fruit SSC content, the ancient cultivars were subdivided into three groups: DD, DS, LG and R cultivars—fruit had with a significantly higher value of SSC (mean value of 16.5° Brix) than fruit from other cultivars, RC, DP and SM fruit were grouped in the middle with a mean SSC value of 13.9° Brix and, finally, fruit from B and DG cultivars with lower\ had\ the lowest\ values\ (12.1° Brix). Values reached by the SSC values of fruit.
from B and DG trees were lower than those of commercial apples cultivars that usually range between 13 and 20° Brix (Drogoudi, Michailidis & Pantelidis, 2008; Iacopini et al., 2010).

### 3.2. Polyphenolic component

Twenty-seven phenolic compounds from five main groups (flavonols, flavanols, anthocyanins, dihydrochalcones and phenolic acids) were found fruit of the ancient apple cultivars and subdivided into five groups (flavonols, flavanols, anthocyanins, dihydrochalcones and phenolic acids) (Table 2). The most common compounds were from Among these groups, the most represented were the flavanol (catechin, epicatechin, procyanidin B1, procyanidin B2, procyanidin B3 and procyanidin B4) and the phenolic acid group (chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, p-coumaroyl glucose, p-coumaroylquinic acid, gallic acid, caffeoyl glucoside, protocatechuic acid, feruloyl glucose and t-cinnamic acid) with differences among cultivars. Finally, among The only anthocyanin, cyanidin-3-O-galactoside, was only found in the fruit flesh from one cultivar (DS), only cyanidin-3-O-galactoside was detected in the cultivar DS. In agreement with the literature, the main compounds found in fruit from this study were chlorogenic acid and epicatechin and this has been previously found in commercial apple cultivars (Chen, Zhang, Li & Ma, 2012; Bi, Zhang, Chen, Zhang, Li & Ma, 2014) (Chinnici, Bendini, Gaiani & Riponi, 2004; Iacopini et al., 2010) and other studies of ancient cultivars as found in commercial (Chen, Zhang, Li & Ma, 2012; Bi, Zhang, Chen, Zhang, Li & Ma, 2014) and ancient cultivars (Iacopini et al., 2010; Panzella, Petriccione, Rega, Scortichini & Napolitano, 2013; Kschonsek, Wolfram, Stöckl and Böhm, 2018). It has been observed that ancient cultivars in many cases account for higher level of total phenolic and in particular chlorogenic acid (Kschonsek, Wolfram, Stöckl and Böhm, 2018).

The total polyphenolic concentration (TPC) in fruit of eight (B, DP, DG, LG, SM, DD, DS, and R) out of the nine cultivars studied ranged from 703.0 to 1201 µg g⁻¹ FW (Table 2), the total polyphenols content (TPC) ranged from 703.0 to 1201 µg g⁻¹ FW—which is higher than the TPC
values found in fruit from other commercial cultivars such as Golden Delicious, Granny Smith and Red Delicious (Tsao, Yang, Young & Zhu, 2003; Kalinowska, Bielawska, Lewandowska-Siwkiewicz, Priebe, Lewandowski, 2014; Remorini et al., 2015; Masi et al., 2017) (Table 2). However we are aware that the comparison between the results of the present experiment and those of obtained in this work to those of other studies might be misleading given that, as phenolic composition of apple fruit can also be influenced by agronomic and environmental, edaphic and more in general and pedo-climatic conditions factors (Awad et al., 2001; Treutter, 2001). To overcome the confounding effects of environmental factors over the genetic background of cultivars. Only the screening of commercial and ancient cultivars would need to be carried out under grown in the same experimental conditions would avoids the possible confounding effects of environmental factors over the genetic background.

To highlight determine the similarities and the differences in the polyphenolic profile among cultivars, in terms of regarding polyphenol profile, in Figure 1 a heatmap, which represents with different colours of the four major phenolic groups found in the apple flesh of fruit from each cultivar and a cluster analysis are shown in Fig. 1. The cluster analysis divided the nine cultivars into three clusters by choosing a relatively large and safe cutting value at the linkage distance of 10. The first cluster was composed by a large part of the cultivars (R, DP, B, DD and SM), the second cluster consisted of was composed by cultivars LG and DS cultivars, while the last group was composed by RC and DG cultivars belonged to the third group cluster. In terms of polyphenols composition, the first cluster was generally characterized by fruit with a high flavanol content in flavanols and a low amount concentration of phenolic acids with the exception of fruit from the cultivar B, which also associated had a high level of dihydrochalcones and phenolic acids as well as a high flavanol content even high level of dihydrochalcones and phenolic acids. Cultivars from To the second group cluster were characterized by belonging cultivars with a high content of phenolic acids and a low content in flavanols in fruit, whereas the last cluster of cultivars was
characterized by cultivars produced fruit with both a low content in flavanols and a low phenolic acid contents.

To investigate or unveil or examine deeper the differences in fruit polyphenolic compositions which allow underpinned the separation of the cultivars to be separated into the clusters shown in Fig. 1, a principal component analysis (PCA) on the polyphenol profile was performed (Fig. 2). The first two principal components (PC1 and PC2) accounted for 57.80 and 33.72% of the total variance in the original data. In the loading plot (Fig. 2a), the first dimension was determined principally consists or was mainly constituted by epicatechin, procyanidin B3 and B2 concentrations, whilst primarily chlorogenic acid concentration determined the originates formed the second component with positive scores indicating high concentrations of compounds. As shown in the score plot (Fig. 2b), there has been a considerable differentiation among-between apple cultivars in the four groups. As shown in the score plot (Fig. 2b), cultivars R, DP, SM and DD showed positive scores for PC1 and negative scores for PC2, except for B cultivar that showed a positive score for both PC the components. A negative score in PC2 indicates a low chlorogenic acid content. The cultivars DS and LG had reached positive scores for PC2 and negative scores for PC1, indicating that these two cultivars were characterized by a low flavanols content. The remaining cultivars, DG and RC, were characterized by the lowest content in phenol concentrations according to the with negative scores reached for both principal components. These results generally are in line with support the previous cluster analysis showed in Fig. 1. Although, a discrepancy for the cultivar B has emerged, grouping it separately but still close to cultivars (but closely related) from R, DP, DD and SM in the PCA analysis, due for to a higher content in chlorogenic acid concentrations in fruittent.

Antioxidant analysis capacity

The highest values of antioxidant capacity determined measured by the DPPH assay were found in fruit from the cultivars LG and R (8.56 and 8.31 μmol TE g⁻¹ FW, respectively), whereas the
lowest value was detected in cultivar RC (2.44 μmol TE g\(^{-1}\) FW) (Fig. 3). The cultivars with the greatest antioxidant capacity (LG and R), were localized in different position in PCA score plot (Fig. 2b, score plot), with negative scores for one of the two components (LG for PC1 and R for PC2), reflecting an opposite differences in content concentrations of chlorogenic acid (high in LG and low in R) and flavanols (high in R and low in LG). It is noteworthy, that fruit from

Interesting to note that B cultivar B, with which had a high value in antioxidant capacity (8.13 μmol TE g\(^{-1}\)), had an intermediate content concentrations of both of chlorogenic acid and flavanols (Tab. 2 and Fig. 3).

In Table 3 significant positive correlations are summarized the correlation coefficients for between phenolic compound concentrations for which, following the row, a positive and significant correlation with the and total antioxidant ability/capacity (DPPH) was found; following the row are summarized: procyanidin B1, catechin, epicatechin, procyanidin B2, chlorogenic acid and procyanidin B3. Clearly, the chemical structure of the each phenol compound plays a key role in determining its antioxidant activity (Abbas et al. 2017). Our data The data are in line with similar to other previous works (Stanger, Steffens, Soethe, Moreira & Do Amarante, 2017; Wojdylo et al., 2008), in which procyanidins, catechin, epicatechin and chlorogenic acid largely contribute have a pivotal role for the contribution to the antioxidant activity in apple fruits (Stanger, Steffens, Soethe, Moreira & Do Amarante, 2017; Wojdylo et al., 2008), although some authors have reported that only procyanidins and epicatechin had a significant role in apple peel and juice (Iacopini et al. 2010; Oszmianski, Wolniak, Wojdylo & Wawer, 2007; Tsao, Yang, Xie, Sockowie, & Khanizadeh, 2005; Grzesik, Naparlo, Bartosz & Sadowska-Bartosz, 2018). Clearly, the chemical structure of each phenolic compound plays a key role in determining its antioxidant activity (Abbas et al. 2017). The reactive centres are the aromatic –OH groups (particularly 3’,4’-dihydroxy catechol group) and their activity is enhanced by other substituents (Abbas et al. 2017). Concerning the flavonoid molecule, the antioxidant activity of flavonoid is principally related to the O-dihydroxy groups at the B-ring because of, to (i) the presence of a C 2-3 double bond, (ii)
to the 3- and 5-hydroxy group and, finally, (iii) to the 4-oxo function in the A- and C-rings (Abbas et al. 2017). However, some authors reported that primarily procyanidins and epicatechin, have a significant role in apple peel and juice, being the most important individual antioxidants in apple (Iacopini et al. 2010; Osmianski, Wolniak, Wojdylo & Wawer, 2007; Tsao, Yang, Xie, Sockovie, & Khanizadeh, 2005; Grzesik, Naparlo, Bartosz & Sadowska-Bartosz, 2018).

**Conclusion**

In this study, the phenolic profile and total antioxidant activity of fruit from nine ancient apple cultivars from Garfagnana (Tuscany, Italy) were analyzed. The cultivars belong to a germplasm collection and their local use and consumption has been progressively abandoned and replaced in favour of the (few) modern commercialized cultivars. Our dataset offers clear evidences that ancient apple cultivars should be re-evaluated not only at the local consumption level, but they can represent a peerless source of genetic variability (e.g., in terms of organoleptic features) as well as nutraceutical properties which can proficiently be exploited for large-scale breeding programs. In particular, leveraging on different specific features of ancient cultivars could also be used for different commercial uses, such as juice production, fresh fruit consumption and extraction of targeted metabolites. We are aware that some work has needs to be done to allow for these cultivars to compete economically with the most cultivated current cultivars, but that however the ancient cultivars studied herein showed a higher total flesh polyphenol content compared to the main commercial cultivars such as ‘Golden Delicious’, ‘Fuji’, ‘Pink Lady’ and ‘Royal Gala’ (Masi et al., 2017; Tsao et al., 2003; Veberic, Trobec, Herbinger, Hofer, Grill & Stampar, 2005), and as well as higher antioxidant activity (Maragò et al., 2015). This shows can represent as the added values of these cultivars and promising starting point for future “nutrafruit” production.
Twenty-six phenolic compounds, regrouped in five principal groups (flavonols, anthocyanins, flavanols, dihydrochalcones and phenolic acids), were detected in the nine cultivars and, in a general way, the ancient cultivars showed a higher total polyphenol content in flesh respect to main commercial cultivar such ‘Golden’, ‘Fujii’, ‘Pink Lady’ and ‘Royal Gala’ (Masi et al., 2017; Tsao et al., 2003; Veberić, Trobec, Herbinger, Hofer, Grill & Stampar, 2005), and a higher antioxidant activity (Maragò et al., 2015). The analyses revealed that RC and DG cultivars had a low appeal in terms of organoleptic and nutritional characteristic. The cultivars SM and DD showed excellent organoleptic characteristics (good compromise between fruit size and SSC) and moderate antioxidant capacity that make them suitable for fresh consumption. As regards the remaining cultivars (B, DP, LG, DS and R), they showed a high polyphenol content and excellent antioxidant activity, but some organoleptic features should be improved, making them therefore interesting genetic sources for breeding programs. Furthermore, R cultivar, due to their high SSC and flavanol content, might be used for a juice production with high nutraceutical properties.

**Founding:** This research did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

**Acknowledgements:** we are thankful to Dr. Annette Richardson and Dr. Simona Nardozza (The New Zealand Institute for Plant & Food Research Limited) for their helpful critical comments on the manuscript.

**Conflict of interest:** the authors declare no conflict of interest

**References**


process. *Journal of Food Composition and Analysis*, 23(6), 518–524.

https://doi.org/10.1016/j.jfca.2009.05.004


https://doi.org/10.1016/j.jfca.2015.09.007


19
Legends of the Figures legends

**Figure 1.** Heatmap visualization of the four major phenolic groups detected on fruit from nine ancient apple cultivars from Garfagnana (Italy). The intensity of different colours shown in the bars scale bar at the top of the figure represents the content concentration each of the phenol groups: phenolic acid, flavanols, flavonols and dihydrochalcones (value expressed in μg g⁻¹ FW of fruit) (left side). On the right side of the figure is reported the hierarchical clustering of cultivars determined from according to the phenolic profile of each cultivar is shown. The cultivars are B: ‘Benito’; RC: ‘Rossa di Corfino’; DP: ‘Della Piastra’; DG: ‘Del Giappone’; LG: ‘Lugliese Grisanti’; SM: ‘San Michele’; DD: ‘Del Debbio’; DS: ‘Del Sangue’; R: ‘Ruggine’.

**Figure 2.** Principal Component Analysis (PCA) describing the separation of phenolic compounds on the base of PC1 (57.8% of variation) and PC2 (33.7% of variation) (a), and the separation of each cultivar replicate using a bidimensional plot on their relative amount of each phenolic compounds based on PC1 and PC2 (b). PCA is illustrated using a bidimensional plot in which components 1 and 2 explain 91.52% of data variability. The cultivars are B: ‘Benito’; RC: ‘Rossa di Corfino’; DP: ‘Della Piastra’; DG: ‘Del Giappone’; LG: ‘Lugliese Grisanti’; SM: ‘San Michele’; DD: ‘Del Debbio’; DS: ‘Del Sangue’; R: ‘Ruggine’.

**Figure 3.** Total antioxidant capacity determined by DPPH assay on fruit from nine ancient apple cultivars from Garfagnana (Italy). Each value is the mean of three replicates ± standard deviation. For each phenol, means flanked by the same letter are not significantly different after one-way ANOVA test with cultivars as source of variability following LSD test (P=0.05). The
Table 1. Organoleptic characteristics on fruit from nine ancient apple cultivars from Garfagnana (Italy). Each value is the mean of three replicates ± standard deviation. For each parameter, means flanked by the same letter are not significantly different after one-way ANOVA test with cultivars as source of variability following an LSD test (P=0.05). The cultivars are B: ‘Benito’; RC: ‘Rossa di Corfino’; DP: ‘Della Piasta’; DG: ‘Del Giappone’; LG: ‘Lugliese Grisanti’; SM: ‘San Michele’; DD: ‘Del Debbio’; DS: ‘Del Sangue’; R: ‘Ruggine’.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Apple cultivars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>102.60 ± 19.12&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Height (mm)</td>
<td>53.26 ± 2.50&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Width (mm)</td>
<td>63.78 ± 3.36&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Firmness (kg cm&lt;sup&gt;-2&lt;/sup&gt;)</td>
<td>9.97 ± 2.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SSC (°Brix)</td>
<td>12.13 ± 0.55&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 2. Profile of phenolic compound concentrations (µg g⁻¹ FW) in fruit from nine ancient apple cultivars from Garfagnana (Italy). Each value is the mean of three replicates ± standard deviation. For each phenol, means flanked by the same letter are not significantly different after one-way ANOVA test with cultivars as source of variability following LSD test (P=0.05). The cultivars are B: ‘Benito’; RC: ‘Rossa di Corfino’; DP: ‘Della Piastra’; DG: ‘Del Giappone’; LG: ‘Lugliese Grisanti’; SM: ‘San Michele’; DD: ‘Del Debbio’; DS: ‘Del Sangue’; R: ‘Ruggine’.

<table>
<thead>
<tr>
<th>Polyphenols</th>
<th>Ancient apple cultivars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
</tr>
<tr>
<td>Flavonoids</td>
<td></td>
</tr>
<tr>
<td>Q-galactoside</td>
<td>0.26 ± 0.15³</td>
</tr>
<tr>
<td>Q-glucoside</td>
<td>1.06 ± 0.97³</td>
</tr>
<tr>
<td>Q-arabinoxyranoside</td>
<td>0.45 ± 0.07³</td>
</tr>
<tr>
<td>Q-arabinofuranoside</td>
<td>0.72 ± 0.22³</td>
</tr>
<tr>
<td>Q-ramnoside</td>
<td>1.56 ± 0.97³</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.03 ± 0.02³</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>4.05 ± 1.92³</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td></td>
</tr>
<tr>
<td>Cyanidin 3-O- galactoside</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Flavonols</td>
<td></td>
</tr>
<tr>
<td>Catechin</td>
<td>52.13 ± 8.19³</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>329.84 ± 60.59³</td>
</tr>
<tr>
<td>Procyanidin B1</td>
<td>13.72 ± 1.96³</td>
</tr>
<tr>
<td>Procyanidin B2</td>
<td>15.38 ± 2.9³</td>
</tr>
<tr>
<td>Procyanidin B3</td>
<td>134.24 ± 20.36³</td>
</tr>
<tr>
<td>Procyanidin B4</td>
<td>3.55 ± 1.15³</td>
</tr>
<tr>
<td>Total</td>
<td>548.86 ± 59.35³</td>
</tr>
<tr>
<td>Dihydrochalcones</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>151.68 ± 25.20³</td>
</tr>
<tr>
<td>Phenolic acids</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlorogenic acid</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td></td>
<td>420.40 ± 35.98&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>246.79 ± 31.86&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>241.43 ± 7.95&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>279.22 ± 6.93&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>487.86 ± 4.21&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>300.47 ± 18.80&lt;sup&gt;ad&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>330.45 ± 31.33&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>574.27 ± 56.07&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>283.91 ± 30.6&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 3. Pearson’s correlation coefficient between phenols-polyphenol concentration and antioxidant the antioxidant capacity (DPPH) on fruit from nine ancient apple cultivars from Garfagnana (Italy). The cultivars are B: ‘Benito’; RC: ‘Rossa di Corfino’; DP: ‘Della Piastra’; DG: ‘Del Giappone’; LG: ‘Lugliese Grisanti’; SM: ‘San Michele’; DD: ‘Del Debbio’; DS: ‘Del Sangue’; R: ‘Ruggine’ **: P<0.01; ***: P<0.001

<table>
<thead>
<tr>
<th>Phenolic compound</th>
<th>Correlation coefficient correlation</th>
<th>Polyphenols content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic acid</td>
<td>0.53**</td>
<td></td>
</tr>
<tr>
<td>Catechin</td>
<td>0.60***</td>
<td></td>
</tr>
<tr>
<td>Epicatechin</td>
<td>0.60***</td>
<td></td>
</tr>
<tr>
<td>Procyanidin B1</td>
<td>0.85***</td>
<td></td>
</tr>
<tr>
<td>Procyanidin B2</td>
<td>0.54**</td>
<td></td>
</tr>
<tr>
<td>Procyanidin B3</td>
<td>0.53**</td>
<td></td>
</tr>
</tbody>
</table>
Declaration of statement

DR, ELP, LG, ML, and RM designed the experiments. ELP and ELP executed the experiments. ELP, GC and ML analyzed results. DR, GC, LG, and RM discussed results and conclusions of the study. ELP wrote the manuscript. DR, ELP, LG, ML, and RM edited manuscript drafts.
Figure 2a
Click here to download Figure(s): Fig 2 a PCA loading .pdf
Figure 3
Click here to download Figure(s): Fig 3.pdf

Total antioxidant activity (µmol TE g\(^{-1}\) FW)

- B
- RC
- DP
- DG
- LG
- SM
- DD
- DS
- R

Ancient apple cultivar