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Comparison of Volatile Organic Compounds, Quality, and Nutritional Parameters from Local Italian and International Apple Cultivars

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Abstract: Apple cultivars 'Annurca' and 'Limoncella' are grown locally in the Campania region of Italy and are valued for their distinctive flavour and characteristics, including a high content of nutritionally important bioactive compounds. However, apples are typically stored chilled for several months before consumption, so it is important to assess if the valuable characteristics are still present after postharvest storage. Here, we compare the quality, nutritional parameters, and aroma of these two cultivars with two widely grown international cultivars, 'Golden Delicious' and 'Fuji', after 60 days of storage. The aroma profiles of all four apples were analysed using thermal desorption and gas chromatography–time-of-flight mass spectrometry. We show that the local cultivars are distinct from the international cultivars in their bioactive compound content and their antioxidant activity. 'Limoncella' shows high sugar content, which may be acting as a cryoprotectant during storage, and high total phenolics in the flesh, which is of nutritional interest. We identified 104 volatile organic compounds (VOCs) and showed that the overall aroma profile is distinct for each cultivar, containing 11 published odorant compounds. The 'Annurca' profile is uniquely low in esters. Seven VOCs retain good discrimination across the four cultivars and, together with the quality and nutritional data, separate the two local cultivars from the international cultivars by hierarchical clustering. Overall, the data emphasize the unique characteristics of the two local cultivars and their value.

Keywords: *Malus domestica*; bioactive compounds; 'Annurca'; 'Limoncella'; 'Golden Delicious'; 'Fuji'

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1. Introduction

Fruit quality encompasses a range of characteristics, including colour, texture, flavour, and health-enhancing compounds [1], all of which develop as the fruit ripens. Apples can continue ripening postharvest, a characteristic typical of climacteric fruits, and can be stored for several months. At the beginning of physiological maturation, apples complete their growth but are not suitable for immediate consumption, as their organoleptic qualities are still evolving through biochemical and structural changes. During the ripening of apple fruit, starch is hydrolysed into simple sugars; the flesh becomes softer due to the depolymerization of components in the middle lamella, such as pectins and hemicelluloses; acidity decreases, polyphenols degrade; and characteristic aromas are formed [2,3]. Apple quality is the result of several factors, including firmness, colour, total soluble solid (TSS) content, and titratable acidity (TA). These factors play a crucial role in assessing fruit ripeness. It is therefore important to determine the optimal harvesting point for apple fruits

by employing various indices such as the starch degradation index, a parameter closely correlated with their physiological ripening. The Thioult index is also widely used for evaluation of the harvest time [4]; moreover, TSS and acidity are key determinants of apple taste characteristics [5]. The initial factor influencing a consumer's decision to purchase fresh fruit is its visual appeal [6,7], represented by various external attributes of the fruit, including its size, shape, absence of imperfections, and colour. In fact, fruit size can also prompt tailored decisions regarding postharvest storage, as excessively large apples may not maintain their quality for extended periods in storage [6]. Consumers generally favour larger fruit; however, this preference is subject to specific limits when it comes to fresh consumption [8,9]. A study conducted in Canada reported the ideal size for dessert apples as ranging between 7.4 and 7.6 cm and 200 g [10,11]; however, this varies across different countries, where smaller fruits are preferred. Another fruit quality parameter is colour, generally represented using the colour coordinates L*, a* (−a for green, +a for red), and b* (−b for blue, +b for yellow). The final red colour of an apple results from the combination of anthocyanins with chlorophyll and carotenoids (background colour) [12].

The nutritional value is also of relevance in this widely consumed fruit. Apple polyphenols are reported to have an important role in disease prevention and to impart beneficial effects on human health [13]. In particular, chlorogenic acid is an antioxidant and an anti-obesity agent [14], epicatechin is reported to prevent and treat intestinal inflammation [15], and catechin has antioxidant activity, which may be its mechanism of action in the prevention of oxidative stress-caused diseases [16]. Procyanidin has antioxidant, anticancer, antitumor, anti-inflammatory, immunosuppressive, and antiallergenic properties, and it also has a protective action against chronic diseases and metabolic disorders [17]. Therefore, assessing variability in individual polyphenols is of relevance to their nutritional value.

The flavour of fruits and vegetables hinges on two key elements: taste, characterized by the harmonious balance between sweetness and acidity, and aroma, which is determined by the concentrations of volatile organic compounds (VOCs). Although the overall flavour profile is the amalgamation of these two factors, aroma is frequently regarded as the primary driver [18]. Given that flavour perception involves the evaluation of numerous VOCs, their thorough assessment is imperative to ensure the selection of high-quality fruits. Yahia [19] and Espino-Díaz et al. [20] identified over 300 volatile organic compounds (VOCs) linked to the fresh flavour and aroma of apples. This extensive array of compounds underscores the complexity that consumers and trained sensory panellists encounter when tasked with describing apple flavour. Widely grown cultivars such as 'Golden delicious' and 'Fuji' have also been studied for the VOC profiles (e.g., Chitarrini et al.; Lee et al. [21,22]), showing common VOC compounds such as hexyl acetate, but overall discrete whole aroma profiles. VOCs detected from whole apples and apple flesh differ. However, a strong correlation has been found between VOCs from intact fruit and their juice [23]. It is desirable to promote the substitution of less flavoured cultivars with those with good aroma and that are also rich in nutraceutical compounds. This can be achieved by harnessing the existing diversity within apple germplasm collections [24]. Examining the physical and biochemical characteristics and aroma profiles of apples holds particular significance in the context of postharvest processing, as the conditions experienced during packing, transportation, and storage can significantly impact the shelf life and the perceived 'freshness' of the product [25,26].

'Annurca' apples are renowned for their organoleptic quality, characterized by a fairly firm flesh and with average juiciness; the flesh is sweet but slightly acidic, with good flavour characteristics. However, the 'Annurca' apple is characterized by the presence of a rather weak stem, which causes the fruits to fall as they grow due to pressure on the branch. During the ripening phase, this process is accentuated, and it is necessary to anticipate harvesting to prevent the fruit from falling to the ground and being damaged. Shortly after being harvested, these fruits undergo a special treatment process to enhance their red colouration. This involves placing the apples on a bed of straw, covering them with shading nets to reduce incident solar radiation and temperature, and manually rotating

them [27]. This is known as a 'melaio' in the Italian region of Campania, which has a long history of cultivation of this apple cultivar. Exposing the fruits to the sun helps promote the oxidation of the anthocyanins present in the peel, turning it into a vibrant red colour. The duration of this treatment spans 15 to 20 days, depending on prevailing weather conditions and the specific characteristics of the fruit. The 'Annurca' apple exhibits excellent post-treatment storage capabilities, retaining firmness [28,29], which has been linked to the pectin composition [30]. 'Annurca' apples were also found to contain a high content of polyphenols compared to 'Red Delicious' and 'Golden Delicious' apples [31], especially in the flesh [32], and large numbers of antioxidant compounds [33]. Previous VOC analyses of whole 'Annurca' apples detected 31 different compounds, noting δ-octalactone, not previously detected in apple VOCs, and high levels of *n*-pentanol [30]. This may be due to the low vapour pressure of these compounds at room temperature. In contrast, when distillates were analysed, 45 VOCs were detected, including a high proportion of esters, and olfactometric analysis found that 2-phenylethanol, β-damascenone, and both ethyl 3- and ethyl 2-methylbutanoate were important components of the aroma [34]. The aroma of 'Annurca' apples is considered to be distinctive and delightful [28,29], although, in one study, its aroma was grouped with less aromatic cultivars [20].

'Limoncella' apples adapt well to hilly and mountainous areas, and the fruit has good resistance to handling and excellent postharvest storage. They are suitable not only for fresh consumption but also for transformation into purees, sweetened or not, for jams, juice, and fruit in syrup. These apples are of medium size with an ellipsoidal shape. Their peel is characterized by a thick, almost waxy texture and with numerous tiny rust-coloured spots [35]. Both the peel and flesh of this cultivar are higher in polyphenols than 'Golden Delicious' and 'Annurca' cultivars [34], with a higher abundance in the peel compared to the flesh [35,36]. The most abundant polyphenols were catechin and epicatechin, which were higher in abundance than in 'Annurca' apples [37]. The flesh of 'Limoncella' apples is relatively firm, although less so than 'Annurca', juicy, and aromatic, with a subtle acidic note, reflected in a high titratable acidity (TA) although not as high as 'Annurca' apples [35,37]. To our knowledge, there are no reports of the VOC profile of 'Limoncella' apples, although this cv. was included in a previous study comparing apple growth origin and type of agricultural practice [38].

Here, we aimed to compare the quality parameters of fruits from two local apples, cv. 'Annurca' and 'Limoncella', with those of two widely grown international commercial cultivars, 'Fuji' and 'Golden Delicious', after a period of postharvest storage. VOC profiles are correlated with the other attributes to highlight the value of the two local cultivars.

2. Materials and Methods

2.1. Experimental Site and Plant Material

This study was conducted in 2022 at a commercial farm located in the province of Caserta (Southern Italy). It involved four distinct apple cultivars: 'Annurca' Rossa del Sud, 'Limoncella', 'Golden Delicious' Clone B, and 'Fuji' SAN-CIV®, which is a cross between Ralls Janet × Red Delicious carried out in Japan (JPN). Descriptions of the four cultivars analysed are reported in Table S1. All the apple trees were ten years old, grafted onto M9 rootstock, and were trained using spindle training systems. They were spaced at intervals of 4.5 m between rows and 1.5 m within rows. This resulted in a planting density of 1481 trees per hectare. Soil was of medium texture, characterized by sufficient levels of both macro- and micronutrients. Irrigation was through a drip system with two self-compensating drippers per plant, delivering a rate of 8 L per hour. The orchard received standard horticultural maintenance, and pest-control measures were implemented in accordance with regulations governing integrated production practices. The environmental conditions (maximum, minimum, and average temperatures) in which the fruits developed are shown in Figure S1.

2.2. Experimental Design

The fruit sampling was performed with four different plants for each cultivar, homogeneous from a vegetative-productive standpoint, in order to obtain 180 fruits to be subsequently divided across the different analyses. All the fruits analysed underwent the same treatment, which involved harvesting them from September until November, +60 days of cold storage in refrigerated rooms at 4 °C with a relative humidity level of 95%. The only exception was for 'Annurca' fruits, which were harvested 15 days before the others to allow the fruits to fully redden in the 'melaio' (Figure S2). To acquire the typical red colouration, 'Annurca' apples were handpicked from the tree and laid out in a 'melaio' to complete the reddening of the fruits. The 'melai' used consisted of small plots of land with a width not exceeding 1.50 m, where the fruits were spread on layers of soft straw or wood chips. The fruits were arranged in rows, exposing the less red side to the light, and were protected from excessive solar radiation with shading nets. At the end of cold storage, 180 fruits per cultivar were collected and used for the various qualitative, bioactive, and VOC analyses.

2.3. Quality Parameters of Fruits

To identify harvest time of fruits, flesh hardness and starch index via the iodine test were determined. In short, ten apples per treatment were sliced horizontally through the middle, and iodine solution was applied to one of the cut halves. After one minute, the Starch–Iodine Index was assessed visually [39]. One of the main processes occurring during apple ripening is the hydrolysis of starch into sugars [40], which begins in the carpel core and concludes in the outer tissues of the cortex [41]. This gradual transformation of starch, visible through iodine staining, serves as a key indicator in determining the optimal harvest time of apples. 'Golden Delicious' and 'Fuji' apples were harvested considering ripening parameters (firmness and starch degradation) previously reported [38], while for the two local cultivars, there are no specific harvesting indices, and reference was made to common agricultural practices. After the period of cold storage (+60 days), the following attributes were examined for all the fruits: weight (g), flesh firmness ($kgcm^{-2}$), epicarp colouration (using CIELAB colour coordinates *L, *a, *b), total soluble solids (TSS) content (°Brix), pH level, and titratable acidity of the juice (TA) expressed as gL^{-1} malic acid. Weight measurements were obtained using an electronic scale, (Precisa Instruments AG, model XB220A, Dietikon, Switzerland) while flesh firmness was assessed using a manual penetrometer (EFFEGI, Model FT 327, Pineridge Rd, Norfolk, United States equipped with an 8 mm tip on two opposing sides of the fruit. Colour measurements were conducted using a colourimeter (Minolta, model CR-400, Tokyo, Japan) on four sides of the apple to ensure sufficient measurements, especially in the case of cultivars with non-uniform epicarp colouration. Both measurements were conducted on 20 fruits. TSS content was determined using a HI 96,814 digital refractometer (Hanna Instruments, Villafranca PD, Italia). pH was measured using a pH meter (Hanna Instruments, Villafranca (PD), Italia), and total acidity was determined through an acid–base titration using a 0.1 N standard solution of sodium hydroxide. To evaluate the ripening stage of the fruits, the Thiault index was also calculated with the following formula: TSS + TA × 10 [4]. All measurements were conducted on four replicates of fruits.

2.4. Extraction and Determination of Polyphenols

Polyphenols from apple flesh and peel were extracted according to the method by Graziani et al. [29], with modifications. Briefly, 0.5 g of lyophilized flesh/peel derived from a quarter of an apple from five fruits for each cultivar was extracted with 5 mL of methanol: water (80:20 v/v). The mixture was vortexed for 1 min. and sonicated in the dark, at room temperature, for 30 min. Subsequently, the solution was centrifuged at 4000 rpm for 10 min. and the supernatants from both extractions were combined and made up to a final volume of 5 mL. The extracts were filtered through 0.22 µm filters. The same extracts were used for measurement of antioxidant capacity and polyphenol profiling via

high-performance liquid chromatography (HPLC). HPLC analysis was performed using an HPLC instrument (Agilent 1200 Series, Santa Clara, CA, USA) equipped with degasser G4225A, DAD detector G1315B, and a Nucleodur C18 reversed-phase column (5 µm, 4.6 mm × 250 mm). The chromatographic conditions were as previously described [29] with modifications as follows. Briefly, the mobile phases were composed of 0.1% formic acid in water (phase A) and 0.1% formic acid in methanol (phase B). Flow rate, 0.5 mL/min; injection volume, 20 µL. The elution gradient was as follows: 0 min, 5% of phase B; 1.3 min, 30% of phase B; 9.3 min, 100% of phase B; 11.3 min, 100% of phase B; 13.3 min, 5% of phase B; 20 min, 5% of phase B. Phenolic compounds were detected at 280 nm for procyanidin B1, procyanidin B2, catechin and epicatechin and 330 nm for chlorogenic acid using a diode array detector (DAD). To quantify the concentration of compounds, calibration curves of standards were constructed. The square of the correlation coefficient (R^2) was 0.992 for chlorogenic acid, 0.990 for epicatechin, and 0.999 for procyanidin B1, procyanidin B2, and catechin. The results are expressed as mg of the phenolic compound/g of dry weight.

2.5. Antioxidant Activity (Flesh and Peel)

The free radical-scavenging activity of the polyphenolic extracts of apple flesh and peel was analysed using 2,2-diphenyl-1-picryl-hydrazyl (DPPH); ferric ion reducing antioxidant power (FRAP) and 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) scavenging. Analyses were performed as previously described [36] with modification. The radical-scavenging activity of the flesh and peel apple extracts was determined by adding 2 mL of DPPH (0.05 mM) radical working solution and 200 µL of diluted extract. The solution was kept in the dark for 30 min., and the absorbance was monitored at 516 nm using a UV-1601PC UV–visible scanning spectrophotometer (Shimadzu, Milan, Italy). For FRAP measurements, the extracts (70 µL) were allowed to react with 279 µL of FRAP reagent and 651 µL of acetate buffer. The absorbance was monitored after 5 min at 593 nm. The ABTS assay was conducted by adding 400 µL of diluted extract and 3 mL of the ABTS working solution. After 5 min, the absorbance was measured at 751 nm using a spectrophotometer. For all three assays, a calibration curve with Trolox as the standard was prepared in the linearity range of 10–200 µM (R^2 = 0.99). The results are expressed as µmol Trolox equivalent per g of dry weight of sample. FRAP, ABTS, and DPPH analyses were performed on three replicates of fruits.

2.6. Collection and Analysis of Volatile Organic Compounds (VOCs)

VOC collection was conducted essentially as described in Muto et al. [42]. VOCs were collected from the headspace of five whole apple fruits from each of the four cultivars treated, as described above. Fruits were placed in sealed nalophan plastic bags (Planit Products Ltd., Malvern, UK) and allowed to equilibrate for 45 min at room temperature. Three replicates were used for each cultivar, and an air control was also included. The headspace (100 mL) was sampled using an Easy VOC hand pump (Markes International Ltd., Bridgend, UK). VOCs were collected directly onto thermal desorption tubes, which were packed with Tenax TA and SulfiCarb sorbents (Markes International Ltd.). Tubes were desorbed as described by Baldwin et al. [43]. Briefly, desorption was carried out at 120 °C for 5 min on a TD100 (Markes International Ltd.) instrument using a nitrogen flow of 40 mL/min, then at 260 °C for 5 min, and were collected onto the focusing trap at 24 °C. Desorption from the trap was at 300 °C (heating rate 24 °C/s) with a trap hold of 5 min and a split ratio of 11:1. Separation of VOCs was performed on a 60 m length, 0.32 mm I.D. and 0.5 µm film thickness Rxi-5ms (Restek, Haywards Heath, UK) capillary column, using He carrier gas at 1 mL/min flow rate into the GC instrument (7890A, Agilent Technologies, Inc., Santa Clara, CA, USA). A temperature programme of 40 °C for 5 min, 10 °C/min ramp to 300 °C, and a final hold of 5 min (total run time 39 min) was applied. A BenchTOF-dx MS (Almsco International, Bridgend, UK) was used to detect VOCs using a source temperature of 275 °C, filament voltage of 70 eV, and m/z range of 35–500 m/z. A retention time standard (C8-C20, Sigma Aldrich, Gillingham, UK) was run with the

samples, prepared by injection of 1 µL of standard mixture directly onto a Tenax TA collection tube. Data processing started with AMDIS (NIST 2014) using MSD ChemStation software (E.02.01.1177, Agilent Technologies Inc.). A custom MS library was produced based on the retention indices, and MS spectra were compared to the NIST library to obtain putative identifications. Identification was based on a >80% mass spectral match both in forward and backwards fit with ±15 retention index compared to the custom library [44]. Non-reproducible compounds (only present in <2 replicates of any sample) were removed from further analysis, as well as compounds also present at comparable levels in the air control sample. Known contaminants from plastics and other sources related to the process were also excluded. Data were normalized to the grand total area abundance for each sample, and a square root transformation was applied, reducing the effect of very abundant compounds in the mixture.

2.7. Statistical Analysis

Chemical–physical and biochemical parameters were compared across the apple cultivars using analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) at a significance level of $p = 0.05$ after verifying that all the data were normally distributed through the Kolmogorov–Smirnov test and testing the homogeneity of the variances through the Levene's test. Where normality or equality of variance were not met, a Kruskall-Wallis test was used instead. ANOVA was performed using SPSS (Statistical Package for Social Sciences) Package 6, version 23. VOC analysis was performed in R software (Ri386 4.2.3) using CAP (Canonical Analysis of Principal coordinates) by applying the function 'CAP-discrim' from the 'BiodiversityR' package, PerMANOVA (Permutational Multivariate Analysis of Variance) by using the adonis' function from the R package 'vegan' and RandomForest™ (RF) by applying 'randomForest'. Heat maps were produced using the R package 'pheatmap'.

3. Results

3.1. Comparison of Physiological and Physical Attributes across the Four Apple Cultivars

All the apple samples underwent assessments of flesh firmness and starch content at the point of harvest to determine the correct stage of ripeness. 'Limoncella' and 'Annurca' displayed higher flesh firmness at harvest, while 'Golden Delicious' and 'Fuji' cultivars exhibited lower values (Table 1). Notably, these results suggest a relationship between greater flesh firmness and higher starch content, as evidenced by the starch degradation values for 'Limoncella' and 'Annurca', which were almost half of the other two cultivars, indicating more starch presence (Table 1), and indeed, correlation analysis revealed an $R^2 = 0.65$.

Table 1. Quality parameters (mean ± SD) of the four apple cultivars at harvest.

Cultivars	Firmness Kg cm^{-2}	Starch Degradation (1–5) *
'Limoncella'	7.2 ± 0.63	1.5 ± 0.11
'Golden Delicious'	6.0 ± 0.50	3.0 ± 0.20
'Annurca'	7.0 ± 0.46	1.8 ± 0.18
'Fuji'	6.5 ± 0.49	3.5 ± 0.22

* 1 = maximum presence of starch; 5 = complete disappearance of starch based on an iodine test.

The quality parameters of the apple samples were then examined following 60 days of cold storage (Table 2).

Table 2. Flesh firmness, fruit weight, colour parameters of the peel (*L, *a, *b), pH, TSS, and TA of four apple cultivars analysed after 60 days of cold storage.

Cultivars	Limoncella	Golden Delicious	Annurca	Fuji	Significance
Firmness (kg cm^{-2})	5.35 ± 0.63 a	4.43 ± 0.50 b	5.20 ± 0.46a	5.38 ± 0.49 a	***
Thiault index	212.83 ± 16.56	190.23 ± 12.21	187.65 ± 16.42	200.15 ± 30.01	ns
Fruit weight (g)	119.58 ± 7.85 d	170.49 ± 12.44 a	145.12 ± 9.97 c	160.45 ± 13.21 b	***
*L	78.60 ± 3.57 a	77.43 ± 3.69 a	33.40 ± 4.50 c	52.25 ± 4.18 b	***
*a	−20.90± 10.96 c	−14.60 ± 11.61 c	77.65 ± 11.58 a	18.18 ± 9.79 b	***
*b	46.13 ± 6.26 b	56.83 ± 7.78 a	7.45 ± 4.05 d	26.35 ± 7.13 c	***
pH	4.01 ± 0.08 a	4.03 ± 0.05 a	3.67 ± 0.13 b	3.98 ± 0.05 a	*
TSS(°Brix)	14.25 ± 1.05 a	13.05 ± 0.42 ab	12.00 ± 1.10 b	13.75 ± 0.69 ab	***
TA (gL^{-1} malic acid)	8.00 ± 0.5a	7.30 ± 0.30 b	8.20 ± 1.17 a	7.40 ± 0.16 b	***

Mean ± SD. The same letter indicates no significant differences between cultivars according to Duncan's multiple range test ($p < 0.05$). Level of significance per the ANOVA is indicated as ns (not significant), * ($0.01 < p < 0.05$), and *** ($p < 0.001$).

The lowest Thioult index value was for 'Annurca', and the highest value was for 'Limoncella'; however, there was no statistically significant difference among the four cultivars Table 2). This indicates that all cultivars exhibited the same stage of ripening. After the 60-day period in cold storage, flesh firmness had diminished across all apple cultivars from an overall mean of 6.68 to 5.09 $kgcm^{-2}$. Following storage, 'Golden Delicious' showed significantly lower flesh firmness compared to the other cultivars and 1.2 fold less than 'Fuji', which showed the highest value. Fruit weight showed considerable variability among the cultivars (Table 2). The *L*a*b colour indices (Table 2) also showed a high variability among the cultivars analysed. Higher values of '*a' were observed in the 'Annurca' cultivar, while lower values were found in 'Limoncella' and 'Golden Delicious', which exhibited higher '*b' values. 'Annurca' had a significantly lower pH compared to the other cultivars (Table 2), while the other cultivars did not differ significantly and had pH values between 3.67 and 4.03. TSS, on the other hand, was highest in 'Limoncella' but significantly higher only compared to 'Annurca' (Table 2). TA was highest in 'Limoncella' and 'Annurca', while the 'Golden Delicious' and 'Fuji' cultivars had lower values (Table 2).

3.2. Profile of VOCs across the Four Cultivars

Across the four cultivars, a total of 104 different VOCs were detected. The largest number belonged to the ester family, including 38 non-acetate esters (which included formates, propanoates, butanoates, hexanoates, octanoates, and hexyl tiglate) and 18 acetate esters. Other VOCs included alcohols and aldehydes [11], aromatic compounds [8], ethers (6), three ketones and carboxylic acids, two alkanes and terpenes, one alkene, and one furan (Table S2).

'Annurca' was distinct in having the highest relative abundance of formate, butanoate, and 2-methylpropanoate esters, as well as ethers, alcohols, ketones, carboxylic acids, alkene, and aldehydes compared to the fruit of the other varieties, and relative lower abundance of 2-methylbutanoate, propanoate, and acetate esters (Figure 1). Hierarchical clustering according to the VOC families separated 'Annurca' apples from all the rest, with the closest clustering of 'Fuji' with 'Golden 'Delicious'. Considering specific VOCs, the 'Limoncella' profile showed a relatively high abundance of ethyl acetate (C10) compared to the other cultivars (twofold greater than 'Annurca') and a more similar relative abundance of propyl propanoate (C39) and 2-methylbutyl acetate (C51) to 'Golden Delicious' and 'Fuji' than 'Annurca'. In contrast, it was more similar to the VOC profile of 'Annurca' in its high relative abundance of ethanol (C01) (Supplementary Table S2). When the whole VOC profile was considered together, PerMANOVA showed a highly significant difference in profiles across the four varieties ($p < 0.001$). Random Forest analysis indicated a completely correct classification of the VOC profiles for each cultivar (Figure 2B), although 'Annurca' and

'Limoncella' were the most distinct in the multidimensional scaling plot (Figure 2A). The mean decrease accuracy analysis within Random Forest identified seven VOCs that were most discriminatory across the cultivars (Figure 2C). Using these VOCs, discrimination was still maintained (Figure 2D), although the 'Fuji' profile was not completely discriminated from that of 'Golden Delicious'(Figure 2E). This indicates that these VOCs represent some of the most important differences across the four cultivars. They include two ethers, three esters, and one carboxylic acid.

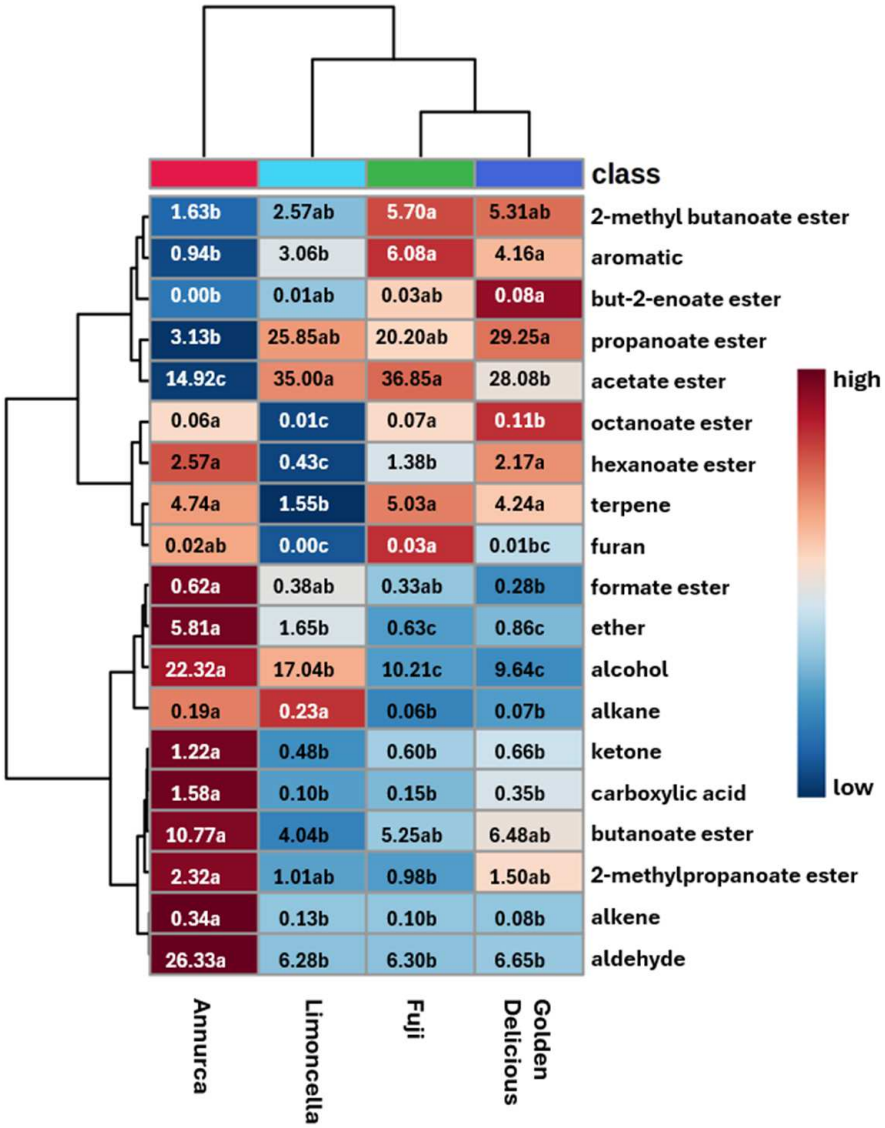

Figure 1. Hierarchical clustering of total relative abundance for each VOC family in the four apple cultivars; mean of three replicates, lettering indicates significant differences based on an ANOVA or Kruskall–Wallis test, $p < 0.05$ amongst the cultivars for each VOC family.

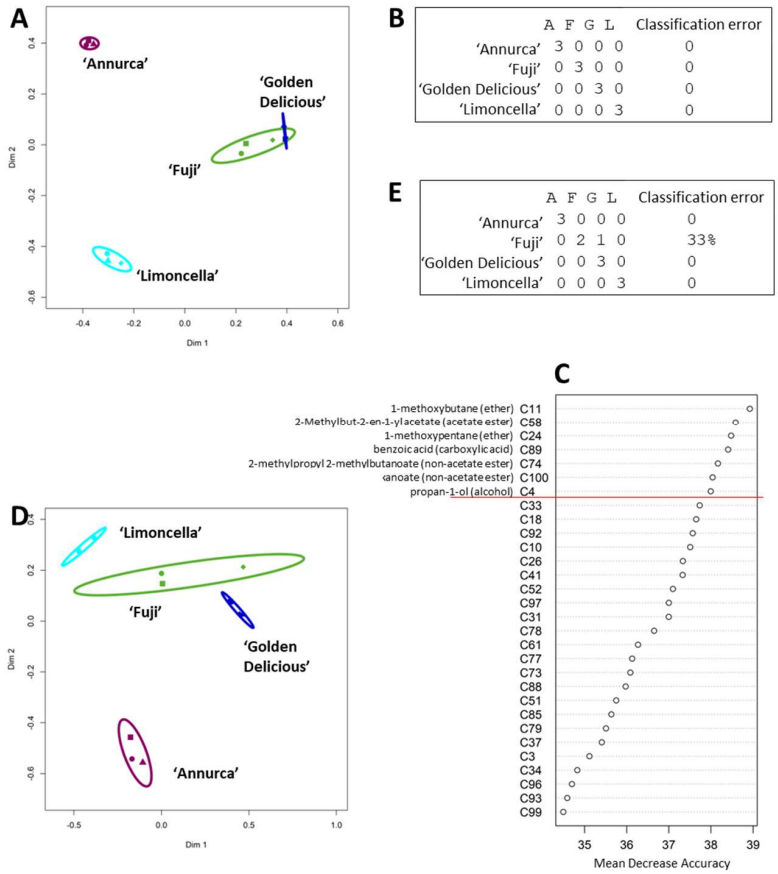

Figure 2. Random Forest analysis of VOCs of the four apple cultivars: 'Annurca', 'Fuji', 'Golden Delicious', and 'Limoncella', (**A**–**C**) using whole VOC profile: (**A**) multidimensional scaling plot, (**B**) classification error, and (**C**) most discriminatory compounds. (**D**,**E**) Random Forest re-run using the top 7 discriminatory compounds (above the red line in **C**), (**D**) multidimensional scaling plot, and (**E**) classification error.

3.3. Bioactive Parameters of the Fruits

The peel of all four cv.s showed higher values of antioxidant activity compared to the flesh measured with the FRAP assay. For both peel and flesh, higher values were reported for 'Limoncella' and 'Annurca' compared to the other two cultivars, with the 'Limoncella' flesh value being two-fold higher than that of either 'Golden Delicious' or 'Fuji' (Table 3).

The DPPH assay did not show significant differences in either peel or in flesh. However, the ABTS assay revealed higher antioxidant activity values in the peel compared to the flesh. In particular, higher concentrations were observed in 'Limoncella' Golden Delicious, and 'Annurca' for the peel, while for the flesh, higher values were reported for 'Limoncella', which had a >3-fold higher value than 'Fuji' (Table 3). Both peel and flesh showed higher values of chlorogenic acid for both local cultivars, 'Limoncella' and 'Annurca', compared to the international cultivars (Table 4).

A similar trend was also observed for catechin. Higher values of procyanidin B2 were found in the peel, particularly in the two local cultivars 'Limoncella' and 'Annurca', which were over 2-fold higher in the peel compared to the flesh, but a high value was also recorded in 'Golden Delicious' peel, also double that of its flesh. The content of total polyphenols was higher in 'Limoncella' and peel compared to the flesh, except for 'Golden Delicious' and 'Annurca', but not in 'Limoncella' and 'Fuji'.

Table 3. Antioxidant activity (DPPH, ABTS, and FRAP) of the flesh and peel of 'Limoncella', 'Golden Delicious', 'Fuji', and 'Annurca' apples analysed after 60 days of cold storage.

Cultivar	FRAP	DPPH	ABTS
	Peel µmol TE/g DW		
Limoncella	57.93 ± 8.01 ab	3.09 ± 0.05	34.24 ± 0.49 a
Golden Del.	46.97 ± 4.43 c	3.39 ± 0.02	34.34 ± 0.27 a
Fuji	50.84 ± 4.00b c	3.50 ± 0.58	30.50 ± 3.51 b
Annurca	60.99 ± 3.63 a	3.00 ± 0.00	32.75 ± 0.50 ab
Significance	***	ns	*
	Flesh µmol TE/g DW		
Limoncella	50.89 ± 3.48 a	3.39 ± 0.02	34.10 ± 0.7 a
Golden Del.	24.46 ± 1.87 c	3.31 ± 0.05	26.24 ± 1.43 b
Fuji	25.40 ± 1.52 c	3.48 ± 0.08	11.14 ± 1.72 c
Annurca	42.71 ± 0.53 b	3.29 ± 0.31	27.05 ± 3.12 a
Significance	***	ns	***

Mean ± SD. The same letter indicates no significant differences between cultivars according to Duncan's multiple range test ($p < 0.05$). Level of significance per the ANOVA is indicated as * ($p < 0.05$), *** ($p < 0.001$), ns (not significant).

Table 4. Individual polyphenols (chlorogenic acid, epicatechin, catechin, procyanidin B1, and procyanidin B2) and total polyphenols (TPC) of the flesh and peel of 'Limoncella', 'Golden Delicious', 'Fuji', and 'Annurca' apples analysed after 60 days of cold storage.

Peel mg/g Dry Weight					
Cultivars	**Limoncella**	**Golden Delicious**	**Annurca**	**Fuji**	**Significance**
Chlorogenic acid	1.26 ± 0.21 a	0.65 ± 0.05 b	1.25 ± 0.05 a	0.77 ± 0.03 b	***
Epicatechin	1.20 ± 0.14 b	1.21 ± 0.07 b	1.48 ± 0.10 a	1.13 ± 0.21 c	*
Catechin	0.63 ± 0.01 b	0.10 ± 0.02 d	1.19 ± 0.14 a	0.30 ± 0.06 c	***
Procyanidin B1	0.42 ± 0.10 a	0.28 ± 0.02 b	0.30 ± 0.00 b	0.06 ± 0.00 c	***
Procyanidin B2	2.09 ± 0.12 b	2.35 ± 0.19 a	1.95 ± 0.19 b	1.09 ± 0.15 c	***
∑polyphenols analysed	5.60 ± 0.16 b	4.58 ± 0.22 c	6.18 ± 0.17 a	3.35 ± 0.24 d	***
Flesh mg/g Dry Weight					
Cultivars	**Limoncella**	**Golden Delicious**	**Annurca**	**Fuji**	**Significance**
Chlorogenic acid	2.39 ± 0.39 a	0.83 ± 0.05 c	1.33 ± 0.08 b	0.93 ± 0.20 c	***
Epicatechin	1.00 ± 0.06 a	0.67 ± 0.11 b	1.03 ± 0.16 a	0.73 ± 0.04 b	**
Catechin	0.95 ± 0.20 a	0.28 ± 0.17 b	0.33 ± 0.08 b	0.11 ± 0.02 b	***
Procyanidin B1	0.98 ± 0.12 a	0.22 ± 0.11 c	0.41 ± 0.10 b	0.19 ± 0.02 c	***
Procyanidin B2	0.95 ± 0.10 ab	1.11 ± 0.08 a	0.86 ± 0.14 b	0.90 ± 0.11 b	***
∑polyphenols analysed	6.25 ± 0.66 a	3.10 ± 0.29 c	2.88 ± 0.32 c	3.95 ± 0.53 b	***

Mean ± SD. The same letter indicates no significant differences between cultivars according to Duncan's multiple range test ($p < 0.05$). Level of significance per the ANOVA is indicated as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), ns (not significant).

3.4. Discrimination of Cultivars and Clustering by Relative Abundance of Discriminatory VOCs, Polyphenol Content, and Anti-Oxidant Activity

Comparing antioxidant activities determined through three assays (ABTS, DPPH, and FRAP), polyphenol values of peel and flesh together and the seven most discriminatory VOCs in a heat map (Figure 3) reveals two clusters: the first one comprises the local apples, 'Annurca' and 'Limoncella', while the second cluster includes the international apples, 'Fuji' and 'Golden Delicious'.

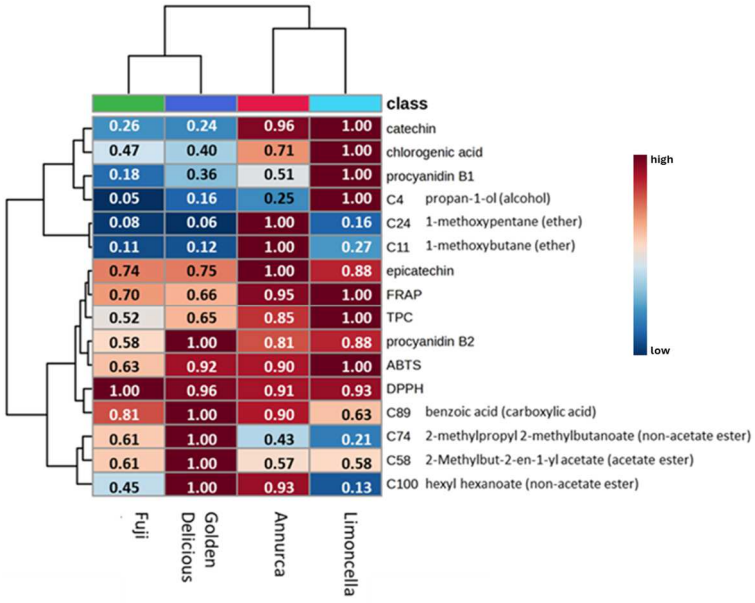

Figure 3. Heat map clustering relative abundance of total polyphenols, individual polyphenols (catechins, epicatechins, procyanidin B1, procyanidin B2, and chlorogenic acid), and antioxidant activities assessed through ABTS, DPPH, and FRAP assays (both flesh and peel) with the top 7 discriminatory VOCs across the 4 apple cultivars examined: 'Limoncella', 'Annurca', 'Golden Delicious', and 'Fuji' (values are normalized to the maximum value for each attribute across the four cultivars).

'Limoncella' and 'Annurca' apples exhibit higher levels of total and individual polyphenols, such as catechins, epicatechins, procyanidins B1, and chlorogenic acid, as well as greater antioxidant activity measured using the ABTS and FRAP methods. On the other hand, the 'Golden Delicious' variety exhibited higher procyanidin B2, while the 'Fuji' variety showed elevated antioxidant activity specifically recorded with the DPPH method.

The hierarchical clustering of the top seven most discriminatory VOCs across the four cultivars (Figure 2) and the phytochemical attributes (Tables 3 and 4) creates two main clusters of attributes (Figure 3). Two ether VOCs (C24 and C11) and the alcohol VOC (C4) cluster with catechin, chlorogenic acid, and procyanidin B1. This cluster has a higher relative abundance in 'Limoncella' or 'Annurca' and a lower relative abundance in the two international cultivars. There were three ester VOCs (C58, C74, and C100) and the benzoic acid (C89) group with the antioxidant attributes epicatechin and procyanidin B2. The second cluster is further subdivided into two groups: the first larger group in which the attributes are generally of relatively lower abundance in 'Fuji', and the second small group where attributes have high relative abundance in 'Golden Delicious' but relatively lower abundance in the other cultivars.

4. Discussion

Apples from all four cv.s were harvested at the optimal time according to common agronomic practice for each variety. The variation in firmness at harvest correlated with starch breakdown, which was cultivar-dependent. Firmness was re-assessed after storage and was more uniform, suggesting a similar stage of ripeness. Indeed, as noted, the Thiault index was uniform across all the cultivars. Apple fruits characterized by excellent quality generally exhibit an index exceeding 180 [4]. In the present study, for all cultivars, this index was >185, suggesting high-quality fruit (Table 2). Fruit weight differed amongst the cultivars and appears to be a distinguishing characteristic (Table 2). All four cultivars were of weights below the reported ideal of 200 g, with the two local cultivars being smaller than the international cv.; however, as also previously noted, fruit size preference varies in different countries [10,11].

Hoehn et al. [45] highlighted that firmness, TSS, and TA are also important indicators of consumer acceptability. Here, the 'Limoncella' cultivar exhibited higher TSS values, in accordance with a previous study [36]. The elevated sugar concentration in this apple variety is noteworthy, impacting both its flavour and perhaps linked to its capacity for extended storage, owing to the cryoprotective properties of sugars. As shown previously [46], 'Golden Delicious' exhibited comparatively lower levels of soluble solids. 'Annurca' TSS values were also in line with those previously reported [36]. 'Limoncella' acidity levels were also similar to those reported [36], while in 'Annurca', lower acidity was detected. The total organic acid content in apples is of significance, not only due to its impact on the eating experience and its association with the flavour and taste of apples but also because organic acids serve as substrates for fruit respiration, both while on the tree and after harvest [47]. If the titratable acidity (TA) is low at harvest, there is a risk that the crop may fall below acceptable quality standards for marketing, as TA tends to decrease during storage [41]. In line with Graziani et al. [29], an intense colouration of the peel was achieved in the 'Annurca' cultivar, attributed to the postharvest treatment in 'melaio'

As reported previously (e.g., Karaman et al. [48]), antioxidant activity measured with ABTS and FRAP and total polyphenols were higher in the peel than in the flesh, except for 'Limoncella' fruits, which showed a significantly higher total phenolics value in the flesh. This is of particular interest for the potential health benefits when the apple is peeled before consumption since a correlation between high antioxidant activity and the inhibition of cancer growth by the apple peel compared to the flesh was previously noted [49].

'Limoncella' or 'Annurca' showed the highest total phenolics, ABTS, and FRAP in both peel and flesh compared to the other two cultivars apart from flesh phenolics in 'Annurca, which were on a par with 'Golden Delicious' and lower than 'Fuji'. This is in agreement with Graziani et al. [36], who compared these attributes across 'Limoncella', 'Annurca', and 'Golden Delicious' and with Tenore et al. [32], who ranked 'Annurca > 'Fuji' > 'Golden Delicious' for total phenolics from both flesh and peel. Thus, the local varieties rank highly in phenolics and antioxidant capacity against the two international cultivars. However, the antioxidant activity appears different depending on the assay used for its analysis. This may be due to the different nature of the radical applied [50] or may be caused by the synergistic effect between polyphenols and other chemical constituents [36]. This is in line with previous studies, where some polyphenolic compounds were shown to have different antioxidant activity depending on the measurement method used [51].

Some of the most important individual phenolics were analysed for their abundance across the four cultivars. In the peel and flesh of all cultivars analysed, chlorogenic acid, epicatechin, catechin, procyanidin B1, and procyanidin B2 showed the highest content in 'Limoncella' and 'Annurca' cultivars, as was reported previously, where these two cultivars were compared to 'Red Delicious' and 'Golden Delicious' [36]. The individual polyphenolic content was generally higher in the peel compared to the flesh, as previously reported [52], although chlorogenic acid and catechin were higher in 'Limoncella' flesh compared to the peel, again also found in other cultivars previously [48,53]. Indeed, the variability in polyphenol content and antioxidant activity appears to be both cultivar- and tissue-dependent, as previously reported in several studies [37,50,54–56].

Few studies to date have reported on the VOC composition of 'Annurca' apples, and to our knowledge, this is the first study of the VOC profile of 'Limoncella' apples. The profile of 'Limoncella' apples was distinct from the other cultivars assessed here and, in particular, distinct from 'Annurca'. An early study using distillate of 'Annurca' apples [34] found 45 different compounds, and in agreement with the data presented here, there was a high representation of esters with many of the individual VOCs shared with the dataset presented here. Another early study using headspace collection from intact fruits, as was performed here, only detected 31 VOCs [30]. The majority of the alcohols and esters detected by Scalzo et al. [30] were also detected here, and 1-pentanol was present, although it was not of very high relative abundance, ranking 37th out of the 104 VOCs detected for 'Annurca'. Unlike the previous study, no lactones were detected. Compared to

the VOC profile from 'Annurca' distillate [34], none of the VOCs identified as important components of the aroma were detected here. Differences may be due to the collection method and sensitivity of the analysis, but also due to the postharvest storage, as the Scalzo et al. [30] study used freshly harvested and 'melaio' ripened apples. 'Golden Delicious' aroma VOCs have also been previously analysed [57], and a total of 36 VOCs were reported, again dominated by esters and alcohols. More recently, Waghmode et al. [58] used SPME to assess changes in VOCs during postharvest storage of 'Golden Delicious' apples, detecting 41 different VOCs at harvest but only 17 VOCs after 60 days of chilled storage. The detection here of over 100 VOCs, even after storage, indicates perhaps the higher sensitivity of the TD-GC-MS-ToF instrument. The analysis of the VOC profiles here highlighted differences in aroma across cultivars, as previously described [24,59,60]. The stage of maturity was found to be an important factor in aroma development in both 'Golden Delicious' and 'Fuji' apples [59] and correlated with the development of sensory-relevant aroma. Thus, it is important when comparing cultivars to ensure parity of maturity, as was achieved here through the starch and other assays.

However, the specific VOCs that discriminate amongst cultivars are likely to differ. For example, Roberts and Spadafora [60] compared four different cultivars: 'Gala', 'Rubens', Grany Smith', and 'Smitten', showing distinct VOC profiles. The VOCs that were found to be most discriminatory by Roberts and Spadafora [60] were different to those found here, although esters were represented in both studies. Farneti et al. [24] compared 190 apple accessions, including 'Golden Delicious', 'Fuji', and 'Annurca', using PTR-ToF-MS and found that Golden Delicious' and 'Fuji' were grouped together away from 'Annurca', as was found here. Farneti et al. [24] also noted the reduced abundance of esters in 'Annurca' compared to the other two cultivars, which seems to be an important characteristic of this cultivar. The clustering of the top seven discriminatory VOCs here with the phytochemical attributes may indicate different metabolic balances across the cultivars and specifically between the two traditional cultivars and the two widely grown international cultivars. The VOCs that have the highest relative abundance in 'Golden Delicious' are all esters, which fits with other studies (e.g., Farneti et al. [24]).

Of the 15 principal odorant compounds listed by Mehinagic et al. [59] in 'Golden Delicious', 'Fuji' and 'Braeburn', and 20 listed by Wu et al. [61] based on 35 apple varieties, 13 were found among the VOCs here. Furthermore, 4 of them were among the 30 most discriminatory compounds across the 4 cultivars analysed here (C37, ethyl butanoate; C51, 2-methylbutyl acetate; C78, hexyl acetate; and C96, hexyl 2-methylbutanoate). This indicates that the differences in aroma reflect likely sensorial differences as well. Three of the top seven discriminatory compounds are described as fruity (The Good Scents Company Information System, https://www.thegoodscentscompany.com/ (accessed on 11 August 2024); C74, 2-methylpropyl 2-methylbutanoate; C100, hexyl hexanoate; and C4, propan-1-ol). They are differentially abundant in the four cultivars: C4 is more abundant in 'Limoncella', C100 in 'Golden Delicious', followed by 'Annurca', while C74 is at higher relative abundance in 'Golden Delicious'. Therefore, the fruitiness aroma of each cultivar may be generated by different VOCs. C89 has been described as having a non-offensive odour and a slightly pungent and acidic taste; this VOC is at higher relative abundance in 'Golden Delicious' and 'Annurca'. In contrast, C11 is not described as an aromatic compound since it is not associated with any particular odour or taste descriptor. The identity of C58 based on the NIST database remains unambiguated: pentyl acetate is described as having a fruity/floral aroma, while there is no sensory descriptor to date for 2-Methylbut-2-en-1-yl acetate. However, C11 has been associated with mechanical damage in 'Golden Delicious' [62]. In our study, it was at higher relative abundance in 'Annurca', followed by 'Limoncella'. In addition, C58 seems to be correlated with consumer-perceived juiciness and acidity in the apple variety 'Smitten', this cultivar being also high in perceived sweetness [61]. In our study, this compound was highly abundant in 'Golden Delicious', where Brix is also high, suggesting a balance of metabolites that may lead to a harmonious aroma and flavour.

5. Conclusions

The VOC profile of 'Limoncella' is distinct from the other cultivars, including 'Annurca'. Aroma profiles discriminate clearly amongst the four cultivars, even after the postharvest storage period, and seven VOCs are identified that retain excellent discrimination. Overall, 'Annurca' is confirmed as having a low relative abundance of esters, while the aroma profile of 'Golden Delicious' is particularly rich in esters. The four cultivars are also distinct in their phytochemical content and antioxidant status, with high antioxidant and phenolic content in the two local cultivars 'Annurca' and 'Limoncella'. These data suggest that the two local cultivars could be of important nutritional value; as such, they are a valuable resource, and it would be interesting to market them more widely. Future work will aim to test how these different profiles are affected by climatic changes in different seasons.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10080863/s1, Figure S1: temperature profile during fruit development; Figure S2: postharvest ripening treatment of 'Annurca' apples; Table S1: origin and description of the four cultivars analysed; Table S2: all VOCs detected across the four apple cultivars.

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