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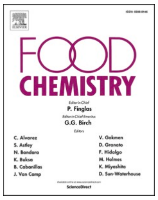

Dissecting the impact of environment, season and genotype on blackcurrant fruit quality traits

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ABSTRACT

This work aims to determine the effect of genotype x environment (GxE) interaction that influence blackcurrant (*Ribes nigrum*) fruit quality. We applied metabolomics-driven analysis on fruits from four cultivars grown in contrasting European-locations over two seasons. By integrating metabolomics and sensory analysis, we also defined specific metabolic signatures associated with consumer acceptance. Our results showed that rainfall is a crucial factor associated with accumulation of delphinidin- and cyanidin-3-*O*-glucoside, the two mayor blackcurrant pigments meanwhile temperature affects the main organic acid levels which can be decisive for fruit taste. Sensorial analysis showed that increases in terpenoid and acetate ester volatiles were strongly associated with higher appreciation score, while proacacipetalin, a cyanogenic-glycoside, was positively associated to bitter taste. Our results pave the way for the selection of high-quality cultivars and suitable production sites for blackcurrant cultivation.

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1. Introduction

Blackcurrant (*Ribes nigrum* L.) berries are appreciated for their high content in bioactive metabolites, their distinct purple-black color and typical flavor. This crop is especially popular and economically important in northern and eastern European countries, with an annual production exceeding 630,000 tons in the European Union (https://www.fao.org/faostat/, 2019). Blackcurrants are consumed both as fresh and processed products (as juices, jams, purees and other food derivatives), and have long been known for their health-promoting properties, playing a protective role against numerous diseases (Abreu et al., 2020; Marsol-Vall et al., 2018). In this sense, fruit metabolic composition, responsible for blackcurrant nutraceutical (phenolic compounds, ascorbic acid) and sensorial (sugars, acids and volatiles) characteristics, has received increased attention (Jarret et al., 2018; Marsol-Vall et al., 2018; Vagiri et al., 2013). Over the last decades blackcurrant genomic resources were developed and with the help of genetic linkage maps and quantitative trait loci (QTL) mapping, genetic factors underlying fruit quality parameters have been outlined (Abreu et al., 2020; Brennan et al., 2008; Hackett et al., 2010; Russell et al., 2014). In blackcurrant fruit, several QTL co-locations were observed between QTLs linked to different phenolic compounds (Abreu et al., 2020) and other quality traits, including anthocyanin derivatives responsible for fruit color (Abreu et al., 2020; Hackett et al., 2010), soluble solid content (Russell et al., 2014), ascorbic acid and juice titratable acidity (Brennan et al., 2008). These findings offer new possibilities for the study of candidate genes and provide essential information for marker-assisted breeding programs of new varieties with enhanced quality attributes.

However, plant breeding is a costly process, being additionally exacerbated in the case of blackcurrant plants due to their perennial nature and the lack of phylogenetically related crop models. Moreover, improving fruit quality traits has long been hampered as a result of the intricate genetics and regulation underlying these traits. However recent advances in metabolomic platforms are facilitating the high-throughput phenotyping of plant metabolic composition (Allwood et al., 2019; Allwood, et al., 2021; Barbey et al., 2021; Pott, Durán-Soria, Osorio, & Vallarino, 2021; Pott, Vallarino, & Osorio, 2021; Vallarino et al., 2018). Previous studies, combining different blackcurrant cultivars with contrasting cultivation sites, have outlined significant effects of genotype and environment, and the interaction of both on phenolic compound composition (Allwood et al., 2019; Tian et al., 2019; Vagiri et al., 2013; Zheng et al., 2012), ascorbic acid content (Vagiri et al., 2013; Walker et al., 2010; Woznicki et al., 2017; Zheng et al., 2009), volatile profiles (Marsol-Vall et al., 2018), sugar and organic acid levels (Woznicki et al., 2017).

However, dissecting the specific effects of environmental and genetic factors on fruit metabolic composition remains a challenging task, additionally hindered by other hard to define variables, such as cultivation practices and ecological processes driven by climate change, among others. Combined multi-platform metabolomic approaches, allowing the identification and quantification of a wide range of quality-associated compounds, with multivariate statistical analyses can shed light on the factors responsible for fruit trait variation. Furthermore, by integrating metabolite profiling with sensory analyses, human perception and liking can be better correlated to a subset of molecules, defining consumer acceptance, and promoting the breeding of cultivars with enhanced quality (Tieman et al., 2017).

The aim of the current study was to investigate the metabolic composition of the ripe fruits of four blackcurrant cultivars (‘Ben Tron’, ‘Ben Tirran’, ‘Ben Gairn’ and ‘Tihope’) by using a combination of gas chromatography- (GC) and liquid chromatography (LC)-mass spectrometry (MS) platforms, and to integrate this information with quality attributes and sensory analyses, to outline chemical preferences. The selection of blackcurrant cultivars was based upon some of the most popular commercially grown cultivars within Europe. Tihope is a Polish cultivar that is widely grown within Poland and neighbouring countries, Ben Tron is a James Hutton Institute (JHI) cultivar that performs extremely well within northern Europe and is a successful commercial cultivar in Norway, whereas Ben Tirran and Ben Gairn, also bred by JHI, perform very well within the UK. In addition, the environmental impact on the measured quality-associated characteristics was assessed, as the same cultivars were grown and harvested in four contrasting European growing sites (Scotland, Norway, Germany, Poland), under similar agricultural practices, and in two successive years, to evaluate seasonal variation. The identification of quality-related metabolites, associated with genotypic or environmental variables, will help future breeding programs and/or cultivation systems to improve berry nutritional and sensorial traits.

2. Material and methods

2.1. Plant material

Plants of ‘Ben Gairn’, ‘Ben Tirran’ ‘Ben Tron‘ and ‘Tihope’ were grown in four different latitudes within distinct European countries: in Geisenheim (Hochschule Geisenheim University, Germany, 49°59́ N, 7°58′ E), in Kapp (Norwegian Institute of Bioeconomy Research, Norway, 60°40′ N, 10°87′ E), in Skierniewice (Instytut Ogrodnictwa, Poland, 51°91′ N, 20°05′ E), and in Invergowrie (JHI, Scotland, 56°46′ N, 3°06′ W), under similar agricultural practice defined by a pre-agreed protocol. Information about the different cultivars is summarized in Table S1.

Fruits were harvested at full maturity, this was defined as when all fruits on a given plant were black, had lost their firmness (had a little give when the fruits were squeezed), and when they had lost the high levels of bitterness indicative of unripe fruit. Fruit development from all four cultivars took similar number of days in each location from open flowers to mature fruit (184/200 days ‘2018‘and 185/203 says´2019́ in Poland; 179/200 days´2018́ and 196/182 days´2019́ in Germany; 205/221 days ´2018́and 220/231 days ´2019́in Norway; 188/200 days ‚2018́and 193/224 days´2019́in Scotland). The planting date occurred during (1) first half of March in Poland and Germany, (2) second half of March in Scotland, and (3) second half of April.

One biological replicate was considered as a pool of approximately 250 g of fully mature fruits of ten individual plants. Each pool was divided into two sub- pools, the first sub-pool (150 g) was ground into a fine power under liquid nitrogen and the second pool were juiced (100 g). All analysis were performed in three biological replicates per cultivar from different seasons (summers 2018 and 2019). For volatile analysis, two biological replicates were used per season.

Meteorological parameters, such as temperature (mean, maximum and minimum), radiation, and precipitation, were recorded daily and are summarized (from flowering to fruit harvest) in Table 1 for 2018 and Table S2 for 2019. Harvest of the fruits took place in July 2018 for the four locations, and in July 2019 (Germany and Poland) and August 2019 (Norway and Scotland).

2.2. Fruit quality attributes: SSC, pH, ascorbic acid, total phenolic compounds, total anthocyanins and antioxidant capacity

For SSC (°Brix), pH, ascorbic acid, total phenolic compounds, total anthocyanins and antioxidant capacity measurements, blackcurrant juice was applied. The fruits were placed in a blender, 0.001 mL of Pectinex 5X (Novozymes) per gram of fruit was added and liquidized for 1 min or until no large pieces of skin could be seen. The liquidized fruit were transferred to a centrifuge bottle and left to settle overnight before centrifuging at 3220 × *g* (Eppendorf 5810R at 4000 rpm (rotor A-4–62) for 20 min at 3 °C. The supernatant was finally filtered through Whatman Number One filter paper.

SSC (°Brix) and pH of the blackcurrant juice was analyzed with a Pocket Brix-Acidity Meter (Atago), three repeat measurements were recorded per each sample, averaged, and expressed as °Brix and pH,

Table 1
Information about the environmental factors of the four locations where the fruits were harvested in 2018, from flowering to harvest. Mean, maximum and minimum temperatures (T) are indicated (°C), being summarized to the average monthly temperature, as well as mean global radiation (W/m^2) and total precipitation (mm^3). GPS coordinates: 51°91′ N, 20°05′ E (Poland), 49°59′ N, 7°58′ E (Germany), 60°40′ N, 10°87′ E (Norway) and 56°46′ N, 3°06′ W (Scotland).

2018	Location	Mean T (°C)	Max. T (°C)	Min. T (°C)	Mean Radiation (W/m^2)	Total Precipitation (mm^3)
April	Poland	13.2	20.6	6.2	4842.3	28.6
	Germany	14.0	20.3	7.7	4705.9	19.4
	Norway	3.5	9.1	−1.2	3835.2	40.4
	Scotland	7.6	11.8	4.1	3297.8	21.6
May	Poland	16.5	24.7	8.0	6092.0	51.6
	Germany	17.5	23.4	11.9	5982.0	71.6
	Norway	15.1	21.5	8.7	5855.7	22.7
	Scotland	12.1	17.5	7.3	5510.9	27.5
June	Poland	18.5	26.2	10.2	5880.7	30
	Germany	20.0	25.1	14.7	6013.3	74.8
	Norway	16.0	21.5	10.5	6196.3	55.8
	Scotland	13.8	19.2	9.6	5531.9	22.8
July	Poland	19.2	25.7	14.0	5080.8	141.4
	Germany	21.4	27.6	15.4	6668.0	3.7
	Norway	20.7	27.1	13.8	6293.5	21.4
	Scotland	16.8	22.0	12.2	5988.9	6.6

respectively.

Ascorbic acid was quantified by high-performance liquid chromatography-diode array detection (HPLC-DAD) at 245 nm according to the methods of Hancock et al. (2007). Calibration was performed against an ascorbic acid reference standard (Sigma Aldrich), the results were calculated as mg/g of fruit.

Total phenolic compounds were determined by a modified Folin-Ciocalteu assay, and total anthocyanins via colorimetric determination, as described in McDougall et al. (2005). Standard curves of gallic acid (Sigma Aldrich) and cyanidin-3-*O*-glucose (ExtraSynthese) were used for quantification, respectively. The total polyphenol results were calculated as mg/mg of fruit, whereas total anthocyanins were calculated as µg/g of fruit.

Antioxidant capacity of the fruits was measured by using two methods, (1) trolox equivalent antioxidant capacity (TEAC assay) as described by Oszmiański and Wojdyło (2007) and (2) the ferric ion reducing antioxidant power (FRAP) assay, in which the antioxidant potential of a sample is measured by its capacity to reduce the Fe^{3+} ion to Fe^{2+}, according to the methods of Payne et al. (2013). The blackcurrant juice sample required a 1:100 dilution in deionized water to bring the sample within the linear calibration range. The FRAP assay was calibrated against 0.25–6 mM of iron sulphate heptahydrate. The results were first calculated as µM of reduced Fe^{3+} per mL of juice before finally being expressed as µM of reduced Fe^{3+} per g of fruit.

2.3. Metabolite profiling

Primary metabolites of whole fruits were measured by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) as described in Vallarino et al. (2018). Identification and quantification of volatile compounds were carried out by headspace solid-phase microextraction coupled to GC–MS (HSPME/GC–MS) as previously described by Pott et al. (2021b). All GC–MS data were relativized to a control sample and to fruit dry weight.

For LC-MS, whole blackcurrant fruits (0.5 g/mL) were extracted as described in Allwood et al. (2019), only with the extraction buffer modified to 99.5 % methanol with 0.5 % formic acid for fresh fruits. Representative samples of each blackcurrant variety were first analyzed by a data dependent HPLC–LTQ-Orbitrap XL MS/MS method as described in Allwood et al. (2019). The LC-MS/MS profiles were applied to confirm the identifications of a wide range of anthocyanins and flavanols, based upon MS/MS fragmentation and retention time matching to reference standards, where obtainable, providing an MSI level 1 identification. The full sample sets were profiled with an Agilent 1260 Infinity HPLC-DAD-6230 TOF/MS (Agilent) under identical chromatographic conditions only applying a lower injection volume of 3 µL. The 6230 TOF/MS system was operated in the 4 GHz high resolution mode (20 K resolution FWHM *m/z* 500) with a scan range of 80–2000 *m/z*, achieving sub 3 ppm mass accuracies by applying a reference mass lock. A full description of TOF/MS parameters is available in Okamoto et al., (2020).

The raw Agilent data files were converted to an MZML format within MassHunter (Agilent), processed through XCMS online (https://xcmsonline.scripps.edu) and subjected to automated annotation procedures and matching to the Manchester Metabolome Database, as described in Allwood et al. (2021). Where LC-MS/MS identifications were not achieved, annotations based upon the accurate mass data were taken under consideration (MSI level 3). The dataset was scaled to the Total Ion Count, prior to being filtered to remove isotope, adduct and fragment ions, preserving only the most intense parent ion feature per each metabolite. Features with greater than 25 % relative standard deviation (RSD), based upon the analysis of replicated quality control (QC) extracts, each prepared from a pool of all blackcurrant fruits, throughout the LC-MS sequence, were filtered. The dataset was finally normalized to the fruit dry weight.

2.4. Sensorial analyses

Sensory analysis was carried out on the juiced (section 2.2) fruits of 'Ben Gairn', 'Ben Tirran' and 'Tihope' harvested in summer 2018 in the four locations. Blackcurrant juices were diluted to a standardized concentration, and ratio of fruit juice to sugar syrup, to provide a consistent material for organoleptic assessment. To define the required volume of juice per each sample, the brix of each juice sample was converted to specific gravity and applied to the following calculation:

$$\text{Juice Volume Required (mL)} = 8.05/\left((\text{Specific Gravity-1}) \times 1000\right) \times 405.$$

Within a 500 mL measuring cylinder, the calculated volume of juice was added, 88 mL of 70° brix sugar syrup was added, and the final volume was adjusted to 500 mL with drinking water. The measuring cylinder was sealed, inverted several times to mix. Then the contents were transferred to clear plastic cups, each coded with a unique three-digit number. Cups were supplied to each taster in a random order to perform the flavor assessment at room temperature in a quiet room supplied with daylight. Seven tasters were selected per a tasting session, all tasters had completed a training course run by a leading UK juice processor to evaluate blackcurrant flavor in juice samples. The tasters were aged between 30 and 60, five were female and two were male, their nationalities consisted of Scottish (n5), Polish (n1), Portuguese (n1). Two of the tasters on the panel had decades of tasting expertise whereas the five other members of the panel had all received training through the experienced tasters. Tasters were supplied with samples, score sheets, a pencil, water and water biscuits for cleansing the palate. The tasters evaluated samples based upon 35 traits linked to four sensorial attributes (appearance, aroma, flavor, and aftertaste), each scored 1–5 (least to most intense) and summed to provide an 'overall appreciation score'. A description of the evaluation of each of the 35 traits is provided in Table S3.

2.5. Multivariate statistical analyses

R software was used for multivariate statistical analysis (https://www.R-project.org/). Hierarchical cluster analysis (HCA) was performed on the mean metabolic values of the three replicates, median-centred and $\log_{10}$-transformed. Pretty heatmap (pheatmap) R package (https://CRAN.R-project.org/package = pheatmap) was used for HCA visualization. Principal component analysis (PCA) was applied using unit variance scaling. One-way ANOVA and Tukey's posthoc tests ($P <$ 0.05) were performed to determine the significantly different sensorial parameters among the three assessed cultivars, for each individual location.

Pearson's pairwise correlations between individual volatiles and sensorial parameters were performed with correlation R package (https://CRAN.R-project.org/package = correlation), using the multi-level correction.

3. Results

3.1. Quality attributes are strongly influenced by the environment and the season

Fully ripe blackcurrant fruits of 'Ben Gairn', 'Ben Tirran', 'Ben Tron' and 'Tihope' were first assessed for important agronomical and quality traits, including fruit size, SSC, pH, total phenols and anthocyanins, antioxidant activity (Ferric Ion Reducing Antioxidant Power, FRAP and Trolox Equivalent Antioxidant Capacity, TEAC) and ascorbic acid

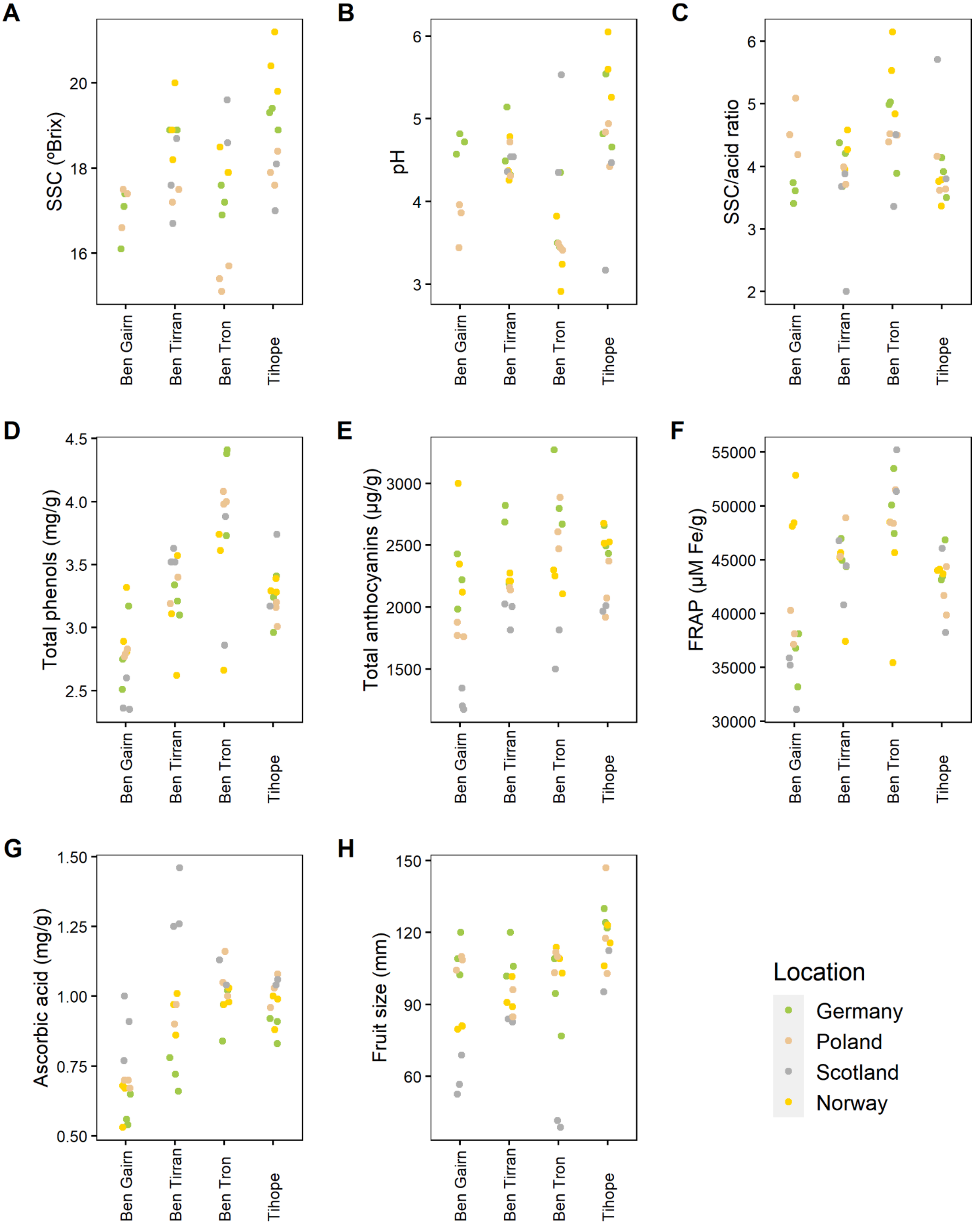

Fig. 1. Soluble solid content (SSC) (A), pH (B), SSC/acid ratio (C), total phenols (D), total anthocyanins (E), ferric reducing antioxidant power (FRAP) (F), ascorbic acid concentration (G) and fruit size (H) of the fruits of the different blackcurrant cultivars harvested in 2018. Each dot represents a biological replicate, with color denoting the location where fruits were harvested.

content (Fig. 1, Fig. S1, Table S4). Differences among the genotypes could be observed and were stable throughout the harvest seasons (2018–2019). For example, ‘Ben Tron’ displayed the highest total phenols (mean value of 3.75 and 3.17 mg/g in 2018 and 2019, respectively) and FRAP antioxidant activity (44400.1 and 41334.5 μM Fe^{3+} per gram of fruit in 2018 and 2019, respectively. Similar behavior was observed by using TEAC assay, Table S4) while ‘Ben Tirran’, together with ‘Ben Tron’ showed enhanced ascorbic acid content, compared to the other cultivars. ‘Tihope’ fruits were larger both years, with a mean diameter of 92.50 and 101.68 mm in 2018 and 2019, respectively (Fig. 1, Fig. S1).

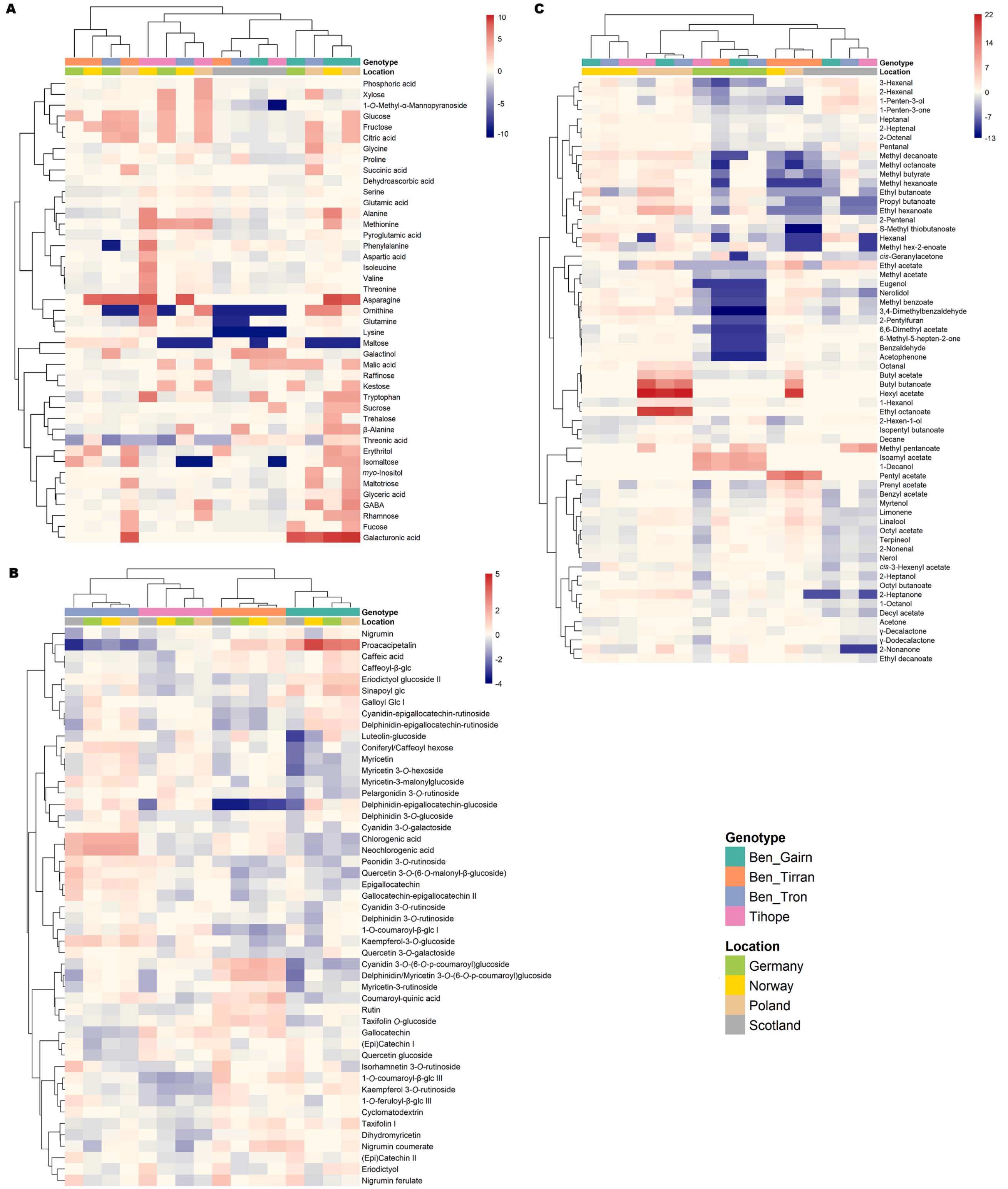

Fig. 2. Hierarchical cluster analysis (HCA) and heatmap visualization of blackcurrant fruits primary metabolites (A), secondary metabolites (B) and volatiles (C) identified in 2018. Each value represents the normalized mean of three biological replicates, with red and blue colors denoting relatively high and low intensities. Metabolites are grouped by clusters, using Pearson correlation coefficients. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

However, notable differences were observed across the cultivation sites and the harvest seasons. The impact of the location on all the measured quality attributes could be noted in both harvest years, however, its effect was not stable across the assessed seasons, possibly as a consequence of variable meteorological conditions in 2018 and 2019 (Table 1, Table S2). For example, the Scottish-harvested samples showed higher ascorbic acid content in 2018 (mean value of 1.09 mg/g) in all the Scottish bred cultivars and lower total anthocyanins (mean value of 1686.8 µg/g) in the four cultivars (Fig. 1), tendencies which were not maintained in 2019 (Fig. S1). In addition, fruits grown in Scotland had the lowest diameter in 2018 (77.65 mm, as a mean value), and the highest in 2019 (123.57 mm). Other striking differences between both years, are the lower pH observed in 2019 (ranging from 2.03 to 4.18) compared to 2018 (ranging from 2.91 to 6.05) in all locations and cultivars, and to a lesser extent, the decrease in SSC in the same harvest (except for Polish-harvested samples), maintaining a relatively stable ratio between soluble solid and acidity (Fig. 1, Fig. S1). FRAP and TEAC antioxidant activity was also decreased in the second year, compared to 2018 in the four growing sites.

3.2. Metabolite profiles are impacted by genetic and environmental factors

Next, metabolite profiling of the blackcurrant fruits was performed to identify and semi-quantify 43 primary metabolites by GC-TOF-MS (Tables S5-6), 49 secondary metabolites by HPLC-MS/MS (Tables S7-8) and 64 volatile compounds by HS-SPME/GC–MS (Table S9-10). While primary metabolites included sugars and derivatives (16), organic acids (nine) and amino acids (19), secondary metabolites were mostly phenolic acids (11), flavonoids (13 flavonols, 11 anthocyanins, five flavanols, three flavanones and one flavone) and five miscellaneous compounds. Among volatiles, the most abundant identified classes were esters (28), followed by aldehydes (12), ketones (eight), alcohols (seven), terpenoids (seven), alkane (one) and furan (one) (Tables S7-S8). Hierarchical cluster analysis (HCA), using Pearson's correlation, was first performed on the normalized mean value of the replicates for primary and secondary metabolites and volatiles to investigate (i) the connection between samples from different genotypes and environments in 2018 and 2019, and (ii) the metabolic relationships among the identified compounds (Fig. 2, Fig. S2). Interestingly, while secondary metabolites allowed sample clustering based on their genotype exclusively (Fig. 2B, Fig. S2B), HCA outlined a combination of genetic and environmental factors impacting primary metabolite and volatile profiles (Fig. 2A,C, Fig. S2A,C). The effect of the environment was particularly noticeable for volatile content in the 2018 harvest, with German-harvested samples clustering together and showing a contrasting pattern in the accumulation of aroma compounds, i.e. higher levels of isoamyl acetate and 1-decanol and lower levels of methyl acetate, nerolidol, 2-pentylfuran, 6-6-dimethyl acetate, 6-methyl-5-hepten-2-one and phenylpropanoid-derived volatiles. Important for the nutraceutical value of the blackcurrant fruits, 'Ben Tron' was characterized by high levels of chlorogenic and neochlorogenic acids, while 'Ben Tirran' showed enhanced content in two anthocyanin derivatives, cyanidin- and delphinidin-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside. On the other hand, the cyanogenic-glycoside, proacacipetalin, was specially increased in 'Ben Gairn' in the two assessed seasons (Fig. 2B, Fig. S2B).

The importance of genotype on secondary metabolite pattern was also highlighted by principal component analysis (PCA, Fig. 3, Fig. S3), with principal component (PC) 1 explaining 25.32 % of the variation in 2018 and allowing cultivar separation, and PC2 (19.76 %) separating Scotland samples from the remaining locations (Fig. 3B). In 2019, PC1 and PC2 (23.93 and 23.21 % of the variation) separated samples based on their genetic origin (Fig. S3B). The two first PC of primary metabolites (25.52 and 18.66 % in 2018 and 34.66 and 19.27 % in 2019) and volatiles (32.78 and 19.76 % in 2018 and 19.55 and 16.53 % in 2019) suggested a more obvious, though partial, separation of the samples based on their geographic origin (Fig. 3A,C, Fig. S3A,C); however, it is worth noting that 'Ben Tirran', based on volatile profile, was clearly separated by PC2 in 2018 from the remaining cultivars and partially by PC1 and PC2 (17 %) in 2019, suggestive of a distinctive aroma pattern (Fig. 3C, Fig. S3C). In addition, HCA and heatmap visualization showed a good clustering of 'Ben Tirran' samples, with remarkably low relative levels of a series of ethyl (hexanoate and butanoate) and methyl esters (decanoate, octanoate, butyrate, hexanoate), and high levels of limonene, linalool, octyl and prenyl acetates, in both years in comparison with the other cultivars (Fig. 2C, Fig. S2C).

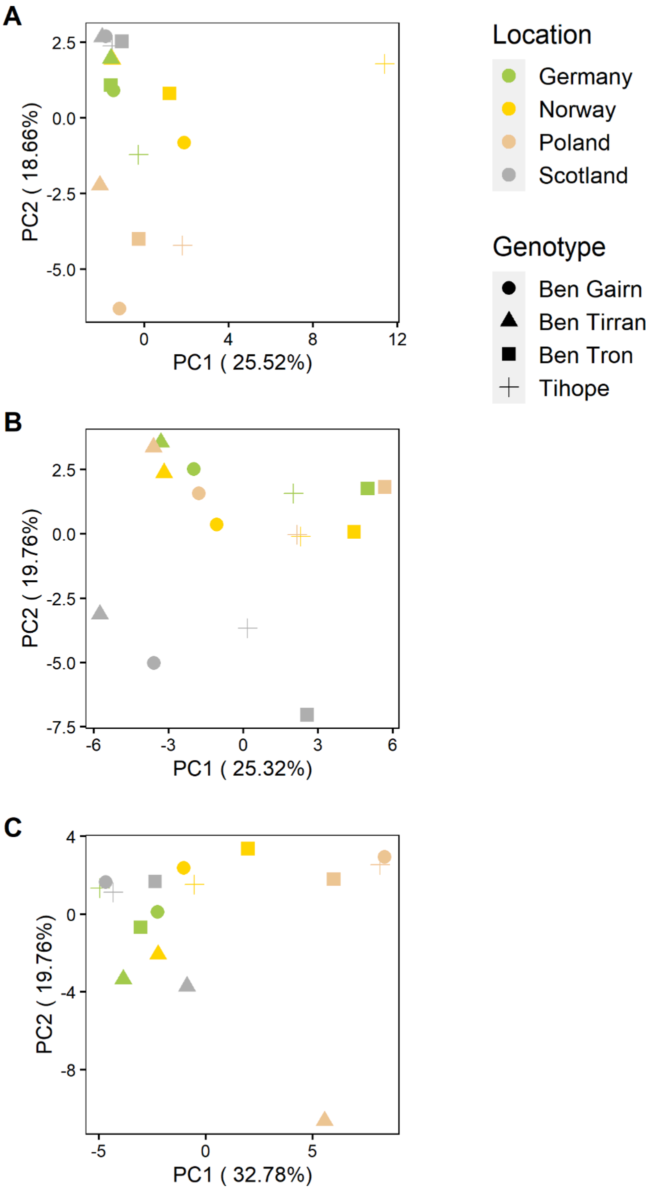

Fig. 3. Principal component analysis (PCA) scores plot showing distribution for primary metabolites (A), secondary metabolites (B) and volatiles (C) of blackcurrant fruits. Each dot represents the mean value of the biological replicates, with shapes indicating the different blackcurrant cultivars, and colors the different locations where the berries were grown in 2018. PC1 and PC2 represents the first and the second principal component, respectively.

3.3. Sensory analyses in 'Ben Gairn', 'Tihope' and 'Ben Tirran'

To link fruit metabolite and quality attributes with consumer liking, a subset of samples, namely 'Ben Gairn, 'Ben Tirran' and 'Tihope' harvested in the four locations during summer 2018, was tested by a trained panel for sensory attributes, and 36 traits related to appearance, aroma, taste and aftertaste were evaluated and scored from 1 to 5 (Table S11). Ten, nine, seven and 12 sensory parameters were significantly different ($P < 0.05$, ANOVA and Tukey's posthoc tests) among the assessed cultivars in Germany, Poland, Scotland and Norway, respectively (Fig. 4). Interestingly, the overall appreciation score was higher for 'Ben Tirran' in the four locations, being significant in three of them (Germany, Scotland, and Norway), while 'Ben Gairn' scored the lowest values. Generally speaking, 'Ben Tirran' scored higher in the significantly different parameters, with the exception of bitter aftertaste in Scotland, higher in 'Ben Gairn' and aroma traits in Norway (also higher in 'Ben Gairn'). Curiously, and although 'Ben Tirran' showed higher overall appreciation in Norway, it also scored higher for parameters such as astringent aftertaste, musty flavor, overall bitterness, and sweaty flavor. Astringent aftertaste was also significantly enhanced in the same cultivar in the Polish-harvested fruits (Fig. 4).

3.4. *A series of volatiles significantly correlate with sensory parameters*

Correlation analyses accounting for the different locations and years using multi-level correction, were drawn between sensory parameters and volatiles, to outline aroma compounds associated with the sensory perception of the fruits. While 52 positive and 106 negative significant correlations were found ($P < 0.05$), only seven correlations showed an r value of around 0.7, indicative of about 50 % of shared variance (Table S12, Fig. 5). Among them, five were positive correlations with the fermented (or vinegary) flavor perception and acetate esters, namely methyl, ethyl, pentyl, prenyl and butyl acetates (Fig. 5). Some of these acetate esters also showed positive associations with sensory parameters correlated with fermented flavor, such as musty flavor, overall bitterness and bitter aftertaste. Sweet aftertaste and overall appreciation clustered together and showed positive correlation with a series of methyl and ethyl esters, although with low r value ($r < 0.6$) meaning that the percentage of shared variance was low (<30 %).

3.5. *Key metabolites for flavor, color and nutritional value are directly affected by meteorological variables*

As agronomical and quality attributes, together with primary metabolite and volatile profiles, were seen to be highly influenced by the growing conditions and harvest year, we aimed to find associations between the measured environmental variables, such as temperatures (mean, maximum and minimum), radiation and precipitation, and some important metabolites or parameters for fruit quality.

During the flowering to harvest (April to July) season, temperatures were generally increased in 2018 compared to 2019 in the four growing sites, with the notable exception of June 2019 for the two southern locations (Poland and Germany). Enhanced acidity was observed in all the

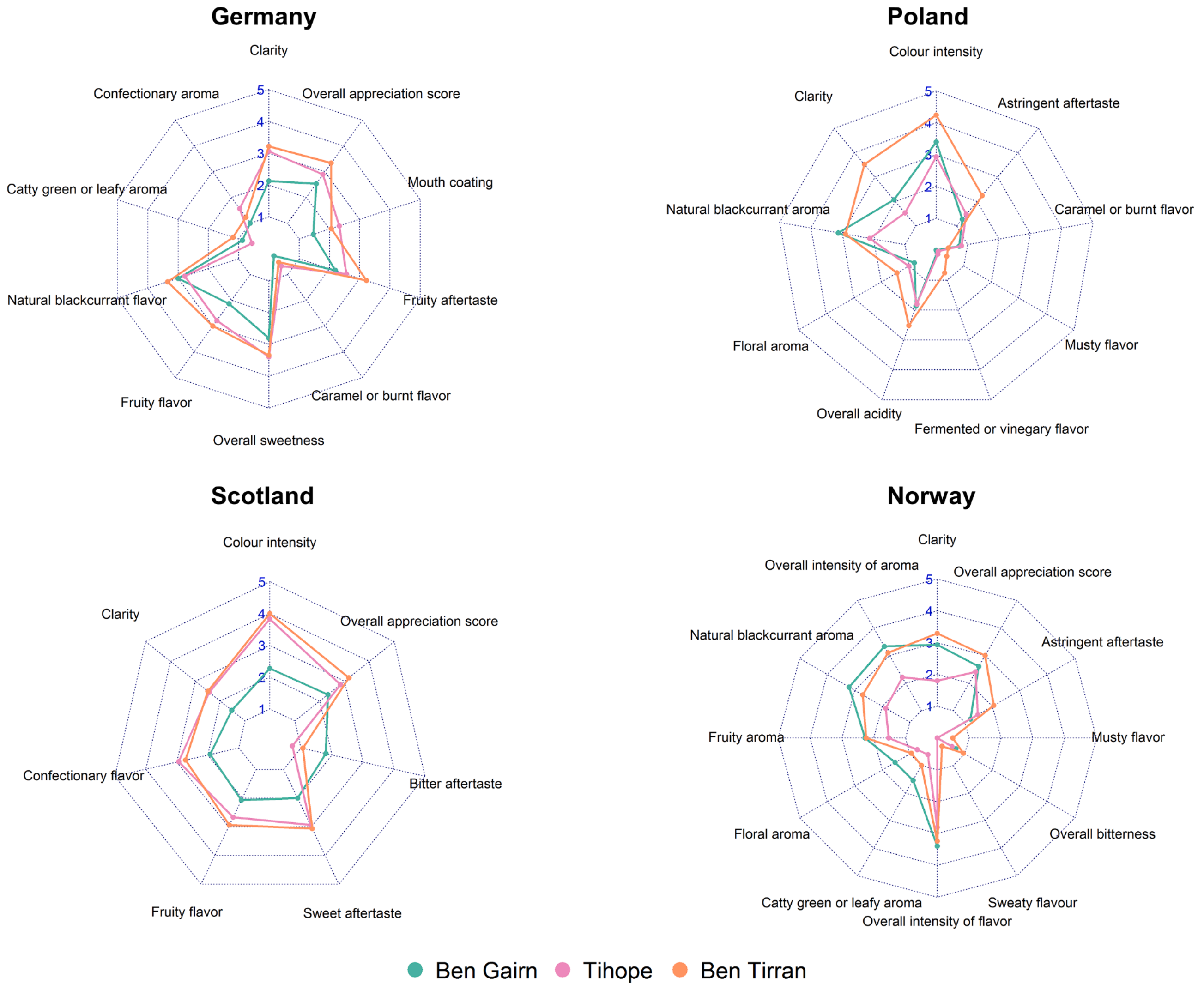

Fig. 4. Radar charts showing the significantly different sensorial parameters among the assessed blackcurrant cultivars ('Ben Gairn', 'Tihope' and 'Ben Tirran') in each location ($P \leq 0.05$).

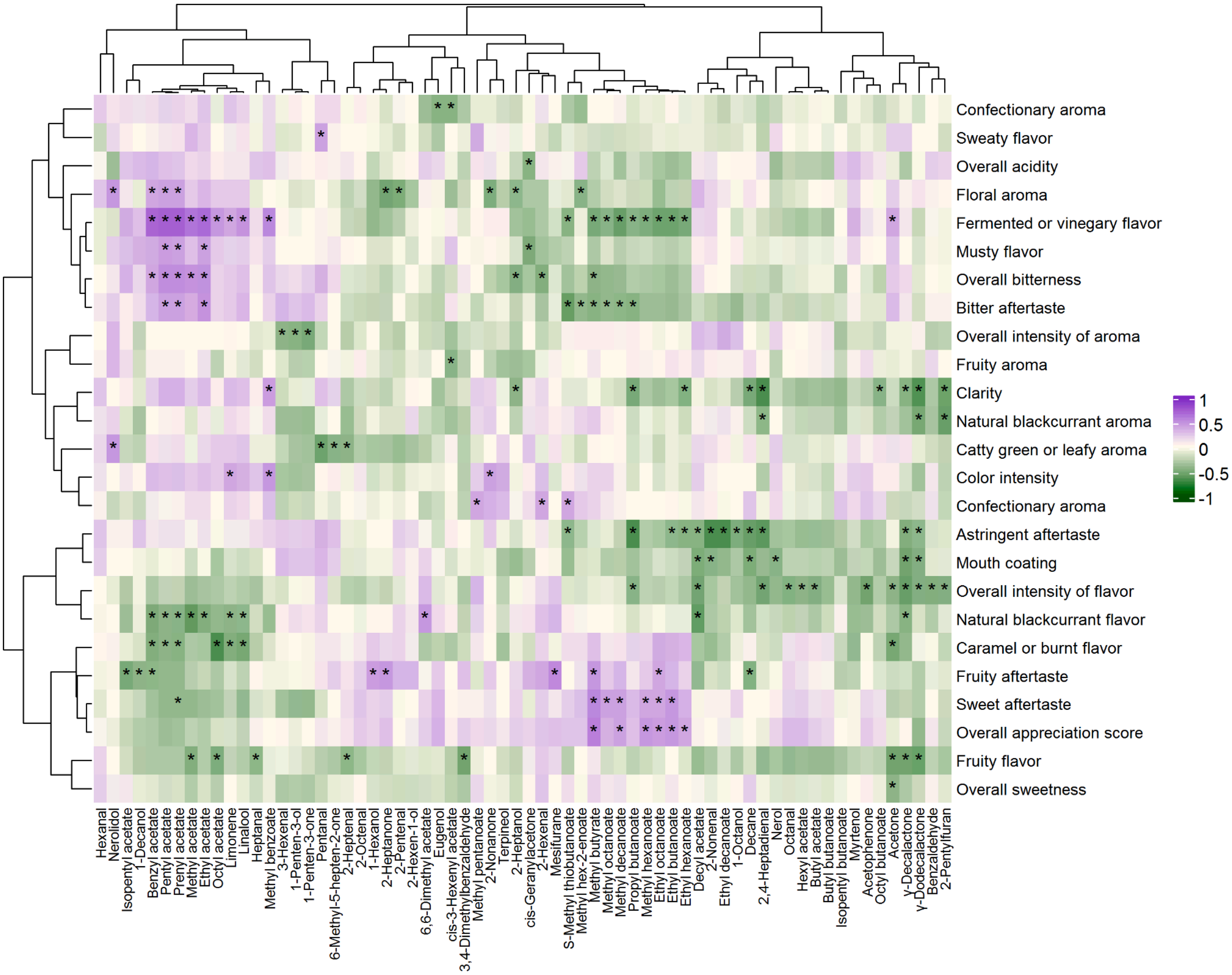

Fig. 5. Heatmap visualization of pairwise Pearson's correlations between volatiles and sensorial parameters, using random factors. Each square represents a given r value in a false color scale (with red and blue colors indicating positive and negative correlations, respectively). Significant correlations ($P \leq 0.05$) are indicated with the asterisk. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

samples in 2019 as shown by the decreased pH (Fig. 1, Fig. S1), and could be explained by higher levels of most organic acids, especially glyceric, succinic, γ-butyric and malic acids; however, it is worth noting that glutamic acid and the major organic acid, citric acid, were decreased in 2019 (Tables S3-4). In addition, a positive correlation between citric acid levels and the difference of temperatures (max. temperature – min. temperature) in 2018 could be drawn for 'Ben Gairn' and 'Ben Tirran' (Fig. S4). However, these correlations were not maintained in 2019 (data not shown).

FRAP antioxidant activity was also diminished in the colder year (2019), compared to 2018, although the major anthocyanin pigments, delphinidin- and cyanidin-3-*O*-rutinosides, which represent more than 60 % of total anthocyanins, one of the most abundant classes of phenolic compounds in blackcurrant fruits, did not appear to be directly affected by temperature. Interestingly, mean temperature in the two last weeks before fruit harvest appeared to have a negative impact on the levels of 1-*O*-coumaroyl-β-glucose III (Fig. S5A), and a positive impact on the levels of the cyanogenic-glycoside proacacipetalin (Fig. S5B) in both harvests (data 2019 not shown), with significant correlations for 'Ben Gairn'. Interestingly, positive correlation was found between proacacipetalin and'Ben Gairn' in 2018 (Fig. S5B).

On the other hand, a positive association could be drawn in 2018 between the levels of delphinidin-3-*O*-rutinoside and total precipitation from flowering to harvest in each location in the three Scottish bred cultivars, with a significant correlation for 'Ben Tirran' (r = 0.99) (Fig. S6A). Furthermore, strong negative association was found in 'Ben Gairn' and 'Ben Tirran' between precipitations in the season 2018 and phenylalanine, the precursor of phenylpropanoid pathway leading to anthocyanin production (Fig. S6B).

Finally, terpenoid volatiles, one of the main classes of metabolites impacting blackcurrant aroma, appear to be increased in the two most southern growing sites, Poland and Germany, compared to the northern locations (Norway and Scotland) and showed globally higher levels in 2018 than 2019, suggesting an impact of the temperature on their levels (Tables S7-8).

4. Discussion

Fruit quality traits, such as flavor, color or nutraceutical value, can be assessed at the metabolite level with the help of high throughput metabolomic platforms and, together with multivariate statistical analyses, the factors responsible for the accumulation of highly valuable compounds can be outlined. In our study, fruits from four genotypes of blackcurrant were grown in contrasting European climates (in terms of latitude and longitude), and analyzed for their content in sugars, acids, phenolic compounds, and volatiles. A combination of genetic and environmental factors was seen to directly impact fruit attributes and metabolic composition, with a strong effect of the growing season, highlighting the need to better define the variables responsible for these quality characteristics.

4.1. Blackcurrant phytochemical profile is influenced by temperature, rainfall, and most prominently by genotype

Secondary metabolites, and in particular phenolic compounds, have been described to be important for the plant interaction with its environment, taking part in defense mechanisms against abiotic stresses, such as light or ultraviolet radiation, or against bacterial and fungal infections (Pott, Osorio, & Vallarino, 2019). Furthermore, modulation of phenolic compound profiles, including flavonoids and hydroxycinnamic acids, was observed under different abiotic stresses such as light, heat and drought (Allwood et al., 2019; Pinasseau et al., 2017; Woznicki et al., 2015a). Specifically, Allwood et al. (2019) described in 'Narve Viking' blackcurrant cultivar and under controlled conditions a negative effect of high temperatures (24 °C) on the accumulation of the main anthocyanins (delphinidin- and cyanidin-3-*O*-rutinoside), as well as quercetin derivatives, suggesting that extreme levels of global warming will also impact functional quality of blackcurrants. However, it is worth noting that under cold climates, such as Nordic (Scandinavian) countries, where average temperatures are unlikely to reach 24 °C, lower latitudes (and thus higher temperatures and radiation) were associated with higher anthocyanin content in blackcurrant fruits (Vagiri et al., 2013). In another study, temperature and radiations have been also described to positively impact delphinidin-3-*O*-glucoside, −3-*O*-rutinoside and myricetin-malonylglucoside in the three same cultivars grown under the same conditions in two distinct Finnish locations (Zheng et al., 2012). In particular, solar radiation levels appear to be a determining driving factor in pigment synthesis, as anthocyanin levels were much higher in blackcurrant fruits grown outdoor under natural conditions compared to controlled growth system (Allwood et al., 2019). Furthermore, natural day-length and photoperiod, which vary significantly along latitudinal gradient, also influenced total monomeric anthocyanin content in four different blackcurrant cultivars, including 'Ben Tron' (Woznicki et al., 2015a, 2016), and could partially explain the higher levels of total phenols, anthocyanins (and FRAP antioxidant value) in 'Ben Gairn' samples harvested in the Norwegian location in summer 2018. Additionally, Marsoll-Vall et al. (2018) observed a negative association between temperature and radiation during the month before harvest and volatile content in three different blackcurrant genotypes, suggesting that global warming due to climate change may negatively impact the sensorial perception of the fruit. The importance of pre-harvest solar radiation was also outlined for ascorbic acid fruit content in Ben Hope cultivar (Walker et al., 2010). Temperature has additionally been described to impact fruit primary metabolism, and in particular the main sugars and organic acids, responsible for fruit taste (Zheng et al., 2009). Notably, exceptional high temperatures during pre-harvest season were seen to negatively affect the levels of these important taste-related metabolites (Tian et al., 2019).

On the other hand, strawberry and blackcurrant fruit antioxidant capacity, which mainly depends on the concentration of phenolic compounds and other antioxidant molecules, such as ascorbic acid, was shown to be positively affected by temperatures (Woznicki et al., 2015b), concomitant with our results, as FRAP values were higher in 2018, the warmer year, than in 2019 for all the cultivars. Total phenolic compounds, which were also increased in 2018 compared to 2019, following the FRAP value trend, showed positive correlation with precipitation during spring and summer in an eight-year experiment including Scottish and Norwegian blackcurrant cultivars, which conclude that cool summer conditions with abundant precipitation is favorable to obtain high yield and quality blackcurrant fruits, enriched in phenolic compounds, anthocyanins and ascorbic acid (Woznicki et al., 2015b). Our results suggest that high temperatures in the two weeks before harvest had a negative impact on blackcurrant nutritional value, by decreasing the levels of the phenolic acid 1-*O*-β-coumaroyl glucose III and increasing the content of proacacipetalin, a toxic cyanogenic-glycoside metabolite. Precipitation should not be seen as water shortage, as the blackcurrant plants were irrigated, when necessary, but as changes in relative humidity, and further impacting other meteorological variables, such as radiation. Furthermore, Zheng et al. (2012) found a positive correlation with the average humidity during the months of March and April and the levels of delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside and myricetin-3-*O*-glucoside. Our data also suggest a positive impact of rainfall on delphinidin-3-*O*-rutinoside in 2018 for the Scottish bred cultivars one of the two main pigments present in ripe blackcurrant fruits, and apparently confirming the importance of rain for blackcurrant nutritional composition. Anthocyanin pigments are the end-products of the flavonoid pathway, and derive from the flavanol dihydrokaempferol, with flavonoid 3′H hydroxylase catalyzing the synthesis of cyanidin derivative anthocyanins and flavonoid 3′5′H hydroxylase the synthesis of delphinidin derivatives (Li et al., 2019). Flavonoids are generated through the general phenylpropanoid pathway, whose precursor is the aromatic amino acid phenylalanine; curiously, a negative correlation between total precipitation and phenylalanine relative levels was observed in the same cultivars where a positive association of this meteorological variable with the main pigments was found, suggesting that specific environmental conditions may drive carbon flux towards the synthesis of anthocyanins.

However, based on HCA and PCA analyses, important differences in phenolic compound profiles could be outlined among the assessed cultivars, allowing sample separation on a genetic basis. This observation confirmed previous studies, where genotype weighed more than the environment on the levels of secondary metabolites in blackcurrants (Vagiri et al., 2013; Woznicki et al., 2016; Zheng et al., 2012) and raspberries (Durán-Soria et al., 2021). Notably, 'Ben Tron' was characterized by high levels of the hydroxycinnamic acids, neochlorogenic and chlorogenic acids, which have been characterized for their beneficial properties on the human health (Lu et al., 2020), while 'Ben Tirran' was enriched in some anthocyanin derivatives (cyanidin- and delphinidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside), the most studied flavonoid class, both for their role in fruit appearance and protection against a broad ranges of diseases (Pott et al., 2019). On the contrary, 'Ben Gairn' presented a relative high amount of proacacipetalin, a cyanogenic-glycoside. Apart from being toxic, this metabolite could also confer to the fruit a bitter taste, as described in some staple crop (Chiwona-Karltun et al., 2004). In fact, the significantly higher bitter aftertaste of 'Ben Gairn' in Scotland, and its generally lower overall appreciation score, could be partially explained by its high content in proacacipetalin, a metabolite which shows a significant positive correlation with bitter aftertaste ($r = 0.57$).

In addition to phenolic compounds, blackcurrant health and antioxidant properties can be attributed to relatively high amount of ascorbic acid (vitamin C), with values in commercial cultivars ranging from 130 to 200 mg/100 mL of fresh juices, matching our data (Vagiri et al., 2013). The effect of the genotype on ascorbic acid content is well known (Raudsepp et al., 2010); for example, 'Ben Gairn' was described as a moderate-to-low ascorbic acid-producing cultivar (Khoo, Clausen, Pedersen, & Larsen, 2012), concomitant with our results. However, a clear effect of the environment was observed in the four cultivars grown in 2018, with higher concentration of ascorbic acid in the samples harvested in Norway and Scotland, and lower value in Germany. A negative correlation between ascorbic acid and temperatures was previously reported in field experiments (Woznicki et al., 2015a, 2015b), in particular with the temperatures recorded in July (Kaldmäe, Kikas, Arus, & Libek, 2013), and our data may suggest that the temperatures reached in July 2018 (greater than20 °C) in the German location were too high for the production of high-quality blackcurrant fruits. In 2019, July's temperatures were lower, including in the German location (<20 °C), and a negative effect of the growing site on ascorbic acid concentration was not observed. Also, previous studies have pointed out that the optimal temperature for blackcurrant cultivation in terms of phenolic compounds and antioxidant compounds was 18 °C (Allwood et al. 2019;

Woznicki et al., 2016). Together, our results confirm that improvement of blackcurrant nutritional value or obtaining specific secondary metabolite profiles for functional food products would be achievable by crossing cultivars with high levels of bioactive metabolites of interest, *i. e.* anthocyanins, hydroxycinnamic acids and ascorbic acid, and favoring climates with high summer precipitation and temperatures around 18 °C.

4.2. Acidity and soluble solid content: Organic acid and sugar trends

One of the most striking differences between the two assessed harvests was the pH decrease in 2019 compared to 2018 in all the cultivars and locations. Concomitant with this observed change in pH values, most organic acid levels were enhanced in 2019, with the notable exception of citric acid, the main contributor to blackcurrant acidity (Tian et al., 2019). Our observation confirmed previous studies in which citric acid content was shown to positively correlate with July temperature (Kaldmäe et al., 2013), and in which elevated post-flowering temperatures caused an increase in this organic acid content (Woznicki et al., 2017). On the other hand, malic acid, the second most abundant organic acid in blackcurrant fruits, showed an opposite trend to citric acid, with increased content in the cooler season. Interestingly, high temperatures induced malic acid decrease, as reported in several species, including blackcurrants and strawberries (Woznicki et al., 2017). Malic acid decrease has been associated with a reduced glycolytic flux and an increase in TCA cycle anaplerotic reactions, suggestive of a poor metabolic efficiency of the fruits under heat stress (Romero, Pott, Vallarino, & Osorio, 2021). Together, the important decrease of pH observed in 2019 could be attributed to the significant increase in malic acid levels in the four assessed cultivars, and may be decisive for fruit taste, as the ratio between main sugars and organic acids is responsible for overall liking (Raudsepp et al., 2010). Furthermore, with the notable exception of 'Ben Gairn', SSC was decreased in 2019 compared to 2018, suggesting that the taste of the fruits harvested in the second year could be negatively affected, as the ratio between sugar and acid is a key parameter for flavor acceptance (Raudsepp et al., 2010). The main blackcurrant sugars (fructose, glucose and to a lesser extent sucrose) were generally decreased in 2019 compared to 2018, and an effect of latitude on their content was clearly observed, with lower latitude locations (Germany and Poland) showing higher relative content. Previous studies highlighted a positive correlation between July temperatures with sugar content, and could explain these differences (Kaldmäe et al., 2013; Zheng et al., 2009). Photosynthetic rate is positively impacted by temperature, leading to sucrose synthesis, which, once transported from leaves to fruits, is hydrolyzed to hexoses (glucose and fructose) by the action of invertases, sucrose synthases, sucrose-phosphate synthase and sucrose-phosphate phosphatase (Zheng et al., 2009).

4.3. Volatiles and sensory parameters

Volatilome of fresh blackcurrant fruits has been previously characterized in several genotypes, with terpenoids, C_6-compounds and esters being the dominant volatile classes (Jung, Fastowski, Poplacean, & Engel, 2017; Marsol-Vall et al., 2018). In particular, monoterpenoids were described to be the prevailing aroma compounds, being indicators of fruit freshness and quality (Chmiel, Kupska, Wardencki, & Namieśnik, 2017). 'Ben Tirran' was characterized for its high content of terpenoids in the two assessed harvests. Curiously, no significant correlations were found between terpenoid compounds and sensory parameters, but it could be hypothesized that the enhanced overall levels of these metabolites may influence the higher appreciation score in this cultivar. 'Ben Tirran' showed significant increased astringent aftertaste, and, more surprisingly, although presenting better overall appreciation, musty flavor, overall bitterness and fermented flavor in some locations; however, with the exception of astringent aftertaste, which is considered a positive parameter for blackcurrant sensorial evaluation, these parameters had very low scores in the three assessed cultivars (<1), suggesting that they were not the dominating perception.

Interestingly, sensory parameters related to fermented flavor, namely musty flavor, overall bitterness and bitter aftertaste, clustered together based upon Pearson's correlations, and were found to positively and significantly correlate with the relative content of several acetate esters (benzyl, pentyl, prenyl, methyl and ethyl acetates). Importantly, ester acetates have been described in grapes as fermentation-derived metabolites (Dennis et al., 2012). Furthermore, ethyl acetate is known as an 'off-flavor' compound, which tends to increase during the postharvest storage of many fruits, including strawberry (Pott et al., 2020) and grape (Maoz et al., 2019), as a result of the activation of the fermentative metabolism. In addition, negative correlation with consumer acceptance has been found in strawberry for pentyl acetate (Ulrich & Olbricht, 2016) and in tomato for prenyl acetate (Tieman et al., 2017). Together, these compounds increased in 'Ben Tirran' compared to the other three cultivars, and may explain the perception of 'fermented', 'vinegary', 'musty' and 'bitter' notes in this genotype; however, we hypothesized that these esters were not found at a sufficient concentration in 'Ben Tirran' to negatively impact its overall appreciation score.

Volatile emission plays a crucial role in the plant's biotic and abiotic interactions with its environment, although the signaling mechanisms remain largely unknown (Pott et al., 2019). A clear effect of the growing location on volatile profiles could be outlined in the blackcurrant samples, more obvious in 2018 than in 2019. Marsol-Vall et al. (2018) concluded that low temperature and radiation during the last month before harvest were associated with a higher abundance of volatiles in blackcurrant; German-harvested samples in 2018, the warmest location, showed particularly low levels of several volatiles, including eugenol, nerolidol, acetophenone, benzaldehyde, and methyl benzoate, among others.

5. Conclusions

Improving fruit quality traits remain a challenging task, due to complexity of their genetics and regulation, and remains an important objective for present and future breeding programs. The results obtained here corroborate the intricate impact of genetic and environmental factors have on fruit flavor and nutritional value. However, visible effects of the genotypes could be observed for important quality-related compounds, such as ascorbic acid, phenolic compounds or terpenoid volatiles, and may provide useful information for breeders. In addition, the consequences of most prominent meteorological variables on important traits and metabolites, such as fruit antioxidant capacity, pH or relative content in citric acid and the main anthocyanins, were discussed and confirmed previous studies on blackcurrant fruits, and may be helpful in the design of guidelines for agricultural practices.

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CRediT authorship contribution statement

Delphine M. Pott: Investigation, Methodology, Resources, Formal analysis, Visualization, Writing – original draft. **Sara Durán-Soria:** Investigation, Methodology, Resources, Formal analysis, Visualization, Writing – review & editing. **J. William Allwood:** Investigation, Methodology, Resources, Formal analysis, Writing – review & editing. **Simon Pont:** Methodology, Resources, Formal analysis. **Sandra L. Gordon:** Methodology, Resources, Formal analysis. **Nikki Jennings:** Resources. **Ceri Austin:** Resources. **Derek Stewart:** Resources. **Rex M. Brennan:**

Resources. **Agnieszka Masny:** Resources. **Anita Sønsteby:** Investigation, Resources, Writing – review & editing. **Erika Krüger:** Investigation, Resources, Writing – review & editing. **Dorota Jarret:** Investigation, Resources, Methodology. **José G. Vallarino:** Conceptualization, Investigation, Supervision, Writing – review & editing. **Björn Usadel:** Investigation, Methodology, Formal analysis, Supervision, Writing – review & editing. **Sonia Osorio:** Conceptualization, Investigation, Methodology, Supervision, Funding acquisition, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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