Metabolic profiling discriminates between strawberry (Fragaria × ananassa Duch.) cultivars grown in Finland or Estonia

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Metabolic profiling analysis with LC-ESI-QTOF-MS was utilized to separate and identify 186 putative major metabolites demonstrating significantly different levels in 15 strawberry cultivars grown in Finland or Estonia. Principal component analysis showed close clustering of genetically related samples grown in Estonia, and hierarchical cluster analyses highlighted differences and similarities in their metabolic profiles driving separation between cultivars with specific metabolic phenotypes. Phenolic acids, flavonoids, flavan-3-ol derivatives, terpenes, and many types of glycosidically bound aroma and flavor precursors showed clear variation between strawberry cultivars. These cultivar-specific differences in the levels of major potentially bioactive phytochemicals in strawberries suggests that cultivar selection is essential for breeding strawberry cultivars with optimal phytochemical compositions contributing to possible functional properties and good cultivation and sensory qualities.

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1. Introduction

In traditional strawberry breeding programs, good disease resistance, storage longevity, and high yield have been among the main objectives (Bestfleisch, Mühring, Hanke, Peil, & Flachowsky, 2014). From the producers’ point of view, firmness, flavor, and shelf life are the most valued traits of strawberry (Yue et al., 2014), while consumers appreciate tastiness and appealing appearance (Rosenfeld & Nes, 2000). In fruits and berries, some classes of phytochemicals influence both the sensory characteristics (e.g. taste, flavor and odor) and the ability to repel biological enemies (Aharoni, Jongsma, & Bouwmeester, 2005; Nieuwenhuizen et al., 2013; Tohge, Alseekh, & Fernie, 2014). For example, strawberry metabolism is to some extent regulated in response to environmental stimuli because many types of plant metabolites are produced in response to biotic and abiotic threats (Crupi, Pichieri, Basile, & Antonacci, 2013; Kårlund et al., 2014a). However, the extent of fluctuation of the strawberry metabolite profile in response to different environmental conditions is often cultivar-dependent (Aaby, Mazur, Nes, & Skrede, 2012; Kårlund, Hanhineva, Lehtonen, Karjalainen, & Sandell, 2015). Strawberry cultivars differ in their qualitative and quantitative metabolite profiles (Aaby et al., 2012; Kårlund et al., 2015), and the metabolic networks determining the development, quality and defense mechanisms of strawberries can be either directly or indirectly modified by breeding (Bestfleisch et al., 2012; Bestfleisch et al., 2014; Ulrich & Olbricht, 2013). However, it has been shown that improvement in disease resistance of fruits may reduce their sensory quality (Coff & Klee, 2006; Nieuwenhuizen et al., 2013).

Strawberries grown in Scandinavia and Baltic states both benefit from the strong image of pure and healthy Arctic berries and berry products owing to the northern climate and low-use of pesticides. The cultivation practices in these countries are generally similar, and the strawberry cultivars that are grown in Finland and Estonia are suitable for cultivation in northern areas, in general. In this study, we surveyed the metabolite profiles of 15 strawberry cultivars grown on commercial farms in Finland or in Estonia to define potential differences in phytochemical composition relevant to consumers, farmers and breeders. Liquid chromatography-based separation of analytes combined with highly accurate and sensitive mass spectrometry technologies provide a modern, universal approach for plant secondary metabolite profiling (Allwood & Goodacre, 2009; Crupi, Genghi, & Antonacci, 2014). In comparison to traditional, targeted approaches, non-targeted metabolite profiling offers a hypothesis-free means for high-throughput examination of small chemical constituents in complex biological samples. In this study, analysis with liquid chromatography connected with
negative electrospray ionization in quadrupole–time-of-flight–mass spectrometry (LC-ESI-QTOF-MS) was utilized to separate and identify major compounds showing significant variation between strawberry sample types. Use of multivariate statistical analyses such as principal component analysis (PCA) and hierarchical cluster analysis was applied to uncover similarities in metabolite profiles among cultivars.

2. Materials and methods

2.1. Strawberries

Samples from 15 garden strawberry (Fragaria × ananassa Duch.) cultivars were collected from commercial strawberry farms in June-July.

Fig. 1. Principal component analysis (PCA) of the metabolite contents of 15 strawberry cultivars grown either in Finland or Estonia. The PCA plot shows differences between strawberry sample replicates according to their metabolite profiles based on metabolite-specific signal abundances. Each replicate is a pool of 10 strawberry fruits. Closely clustered samples grown in Estonia are indicated with a dashed line circle. t1; t2; t3.

Fig. 2. A tentative pedigree of strawberry cultivars Capri (Ca), Clery (Cl), Dely (De), Florence (Fl), Honeoye (Ho), Ischia (Is), Joly (Jo), Jonsok (Jn), Linosa (Li), Marmolada (Ma), Polka (Po), Rumba (Ru), Salsa (Sa), and Sonata (So). Cultivars that were studied are presented in white and blue squares and arranged according to hierarchical clustering (HCL) analysis based on the normalized signal abundances of 1569 strawberry molecular features across all analyzed samples. Parent cultivars Valentine (Val), Senga Sengana (Sen), Induka (Ind), Vibrant (Vib), Gorella (Gor), Holiday (Hol), CIVRI30 (Civ), selection A20–17 (A20–17), selection T2–6 (T2–6), and Elsanta (Els) are presented in circles. Arrows indicate the relatedness between cultivars. Producers of the cultivars that were studied are presented in black-bordered squares on top of the HCL dendrogram. AAFC, Agriculture and Agri-Food Canada; NMBU, Norwegian University of Life Sciences; NYSAES, New York State Agricultural Experiment Station. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
2.2. Non-targeted metabolite profiling

2.2.1. Sample preparation

For the metabolite extraction, 10 frozen fruits (61-152 g) for each cultivar were gently thawed in a microwave oven and homogenized with stick blender. Chemical compounds were extracted from pureed strawberries with 80% methanol (30 mL per 10 g of puree). Suspensions were quickly vortexed and extracted in a horizontal shaker (Unimix 2010, Heidolph Instruments, Schwabach, Germany) at 400 rpm for 15 min at room temperature. Extracts were filtered through a gauze and centrifuged (Centrifuge 5810R, Eppendorf, Hamburg, Germany) at 3220 g for 5 min. The clarified supernatants were stored at -75 °C until analysis. Prior to the metabolite analysis, samples were again centrifuged and filtered (Acrodisc® 0.22 μm PTFE filter, Pall, Port Washington, NY, USA).

2.2.2. LC-MS conditions

The LC-MS conditions have been described in detail earlier (Kårlund et al., 2015). Shortly, the samples were analyzed by LC-ESI-QTOF-MS (Agilent Technologies, Waldbronn, Karlsruhe, Germany) that consisted of a 1290 LC system, a Jetstream electrospray ionization (ESI) source, and a 6540 UHD accurate-mass QTOF spectrometer.

For each strawberry cultivar, three biological sample replicates were analyzed, one replicate being a pool of 10 individual fruits. Two microilters of the sample solution were injected onto an RP column (Zorbax Eclipse XDB-C18, 2.1 × 100mm, 1.8 μm) (Agilent Technologies, Palo Alto, CA, USA) kept at 50 °C. Mobile phases, delivered at 0.4 mL/min, consisted of water (eluent A) and methanol (eluent B) (Sigma-Aldrich, St. Louis, MO), both containing 0.1% (v/v) of formic acid (Sigma-Aldrich, St. Louis, MO). Data were acquired in negative electrospray ionization (ESI-), 2 GHz extended dynamic range mode was used, and the instrument was set to acquire over the m/z 20–1600. For automatic data dependent MS/MS analyses, precursor isolation width was 1.3 Da, and from every precursor scan cycle, 4 most abundant ions were selected for fragmentation. Collision energies were 10, 20, and 40 V in subsequent runs. The sample tray was kept at 4 °C during the analysis.

2.2.3. Data processing

The collection of the data matrix was performed using MassHunter Qualitative Analysis B.05.00 (Agilent Technologies, Palo Alto, CA, USA). Ions were combined to molecular features exhibiting isotopic peaks, dimers, and common adducts. Mass Profiler Professional software (version 2.2, Agilent Technologies, Palo Alto, CA, USA) was used for compound alignment. Noise and low abundance features were removed from the data matrix which was further filtered to qualify only features present in all three sample replicates of at least one sample type with Benjamini-Hochberg corrected p-value of < 0.05 between all samples (analysis of variance, ANOVA). The final dataset contained 1569 molecular features.

k-Means clustering (KMC) analysis with heat map representation (TM4 Microarray Software Suite, available at. www.tm4.org/mev.html; algorithm according to Soukas, Cohen, Socci, & Friedman, 2000) was applied to analyze and display the levels of the differing compounds among all the sample types. The KMC run was conducted in calculated means mode. In the KMC analysis, the molecular features were grouped to 15 clusters representing 44 to 258 molecular features (3-16%) of total 1569. To build a hierarchical dendrogram of sample replicates and types, hierarchical clustering (HCL) was utilized (TM4...
Microarray Software Suite; Eisen, Spellman, Brown, & Botstein, 1998. Principal component analysis (PCA) (Simca 14, Umeå, Sweden) was applied to monitor the relationship between samples and sample replicates based on their metabolite profiles.

For the comparison of the clusters and compound abundances, the data matrix was exported to Excel and sorted based on the maximum peak area abundance value among each cluster. From each cluster, 5 to 27 major compounds were identified on the basis of the fragmentation patterns in the data-dependent MS/MS acquisition. The MS/MS data and the molecular masses were compared against the METLIN Metabolite Database, SciFinder, Dictionary of Natural Products, NIST Chemistry WebBook, and Human Metabolome Database or earlier published work describing fragmentation patterns (Aharoni et al., 2002; Álvarez-Fernández, Cerezo, Cañete-Rodríguez, Troncoso, & García-Parrilla, 2015; Binh et al., 2014; Calani et al., 2013; Engström, 1998; Hanhineva et al., 2008; Hanhineva et al., 2012; Herderich et al., 1997; Kerwin, Wiens, & Ericsson, 1996; Li, Luo, & Kong, 2009; Mena et al., 2012; Ornelas-Paz et al., 2013; Stevenson et al., 2007; Zeng, Xiao, Liu, & Liang, 2006). Altogether, 186 potential metabolites were tentatively annotated.

3. Results and discussion

3.1. Poly- and heterocyclic compounds, acids and lipids predominate in the metabolite profiles of strawberry

In order to determine the cultivar-dependent differences in the metabolic profiles we focused on metabolite signals that were detected in all three replicates of at least one sample type, with significant (p < 0.05) variation between samples. This resulted in dataset of 1569 molecular features, out of which 186 individual compounds were identified. Significant variation between cultivars was found for many distinguishing strawberry metabolite classes, for example flavonoids (e.g. pelargonidin, quercetin, kaempferol and naringenin derivatives, catechin), condensed tannins (e.g. procyanidins and propelargonidins), phenolic acid derivatives (e.g. cinnamic, coumaric, ferulic, ellagic and gallic acids), and hydroxylationates (Table S1). In addition, the non-targeted approach detected some lesser-known compounds that drive the separation between strawberry cultivars, for instance glycosidically bound volatile compounds (e.g. carboxylic acid, benzyl alcohol, and terpene glycosides), hetero- and polycyclic compounds (e.g. lignans), and some other miscellaneous compounds (e.g. peptides, carbohydrate derivatives, phenylethyl cinnamates, quinones).

3.2. The metabolite profiles of strawberries were discriminated mainly according to samples’ genetic background

Principal component analysis (PCA) brought out the variation in the metabolic phenotypes of strawberry samples (Fig. 1). PC1 and PC2 explained 22.6% and 11.3% of the variation, respectively. Notably, all cultivars grown in Estonia except Sonata (i.e. Capri, Clery, Dely, Ischia, Joly, Linosa, and Marmolada) were grouped together in the PCA. In addition, cultivars Florence, Rumba, Salsa, and Sonata showed close aggregation in the PCA plot. Cultivars Jonsok and Honeoye were clearly distinguished from all the other sample types. All samples grown in Estonia, except Sonata, are derived from the same producer, and many of them descend from the same parents (Fig. 2) (Leis, Martinelli, & Castagnoli, 2012a; Leis, Martinelli, & Castagnoli, 2012b; Leis et al., 2012c; Leis et al., 2012d; Leis, Martinelli, & Castagnoli, 2013; Meulenbroek, 2007; Musacchi & Leis, 1994). Hence, the resemblances in the phytochemical contents of the strawberry samples grown in Estonia may be at least partially due to the commonalities in their genetic backgrounds. It is also known that cultivars Rumba, Salsa, and Sonata are produced by Fresh Forward (Fragaria Holland, 2016) and that both Sonata and Florence are descendants of
culturvar Gorella (Fig. 2) (MEIOSIS, 2016; Meulenbroek, 2007). Presently, there is no further information available on the parents of Rumba or Salsa.

The molecular features that drove the separation of strawberry samples in the PCA were classified into 15 clusters by the k-means clustering (KMC) analysis (Fig. 3). Interestingly, obvious clusters containing molecular features that were particularly abundant in one cultivar were found for most cultivars demonstrating the large variability in the phytochemical content between strawberry genotypes and possible cultivar-specific patterns. Molecular features that were abundant in Jonsok, Bounty, Polka, Dely, Florence, Honeoye, Ischia, and Clery were accumulated in clusters 1–6, 8 and 10, respectively. In the hierarchical clustering (HCL) analysis, the samples were clustered primarily according to sample replicates, and some genetically related cultivars were also closely clustered (Fig. 3). For example, cultivars Jonsok, Bounty, and Polka, all descendants of cultivar Senga Sengana (Fig. 2) (Eikemo, Stensvand, Davik, & Tronsmo, 2003) were connected in the HCL dendrogram, and molecular features that were on high level in Jonsok and Bounty were highlighted in cluster 15 (Fig. 3). The molecular features that were accumulated in cluster 15 were on relatively high level in cultivar Polka, as well. In cluster 3, where Polka was the dominant cultivar, Jonsok, Bounty, and Sonata were also slightly distinguished. It is known that Polka is a parent of Sonata (Meulenbroek, 2007), and that Polka is a cross between cultivars Sivetta and Induka, which is a descendant of Senga Sengana (Eikemo et al., 2003) (Fig. 2). Cluster 14 accumulated molecular features that were relatively abundant in Jonsok and Honeoye, two cultivars related to cultivar Valentine (Eikemo et al., 2003).

All cultivars grown in Estonia except Sonata (i.e. Capri, Clery, Dely, Ischia, Joly, Linosa, and Marmolada) were highlighted in cluster 12 in the KMC analysis (Fig. 3). In addition, cultivars Ischia, Capri, Linosa, Clery, and Joly were all closely situated in the HCL dendrogram (Fig. 3), and some molecular features that were on higher level in Capri and Linosa were accumulated in cluster 9. Cultivars Capri, Ischia and Linosa are all descendants of strawberry cultivar CIVRI30 (Leis et al., 2012a; 2012c; Leis et al., 2013).

3.3. Flavonoids, phenolic acids and terpenes showed universal distribution with some cluster- or cultivar-specific trends.

Certain flavonoid derivatives and phenolic acids were observed in almost all clusters (Table S1), and their ubiquity may reflect their fundamental role in plant ecology and secondary metabolism. However, some clusters were characterized by the accumulation of specific classes of flavonoids and phenolic acids. For example, cinnamic acid hexoside derivatives were highlighted in cluster 4 which accumulated molecular features that were on especially high level in cultivar Dely (Table S1; Fig. 3). Cultivar Ischia had the highest levels of two derivatives of aminobenzoic acid anthranilate (cluster 8), which were detected in only a limited number of cultivars: Ischia, Capri, Dely, Jonsok, and Sonata (Fig. 4a). The putative anthranilate glycoside (MW 461.1532) was detected also in Salsa, Marmolada, and Clery.

In addition to anthocyanins, other flavonoid derivatives (querceitrin, kaempferol, dihydrokaempferol, naringenin, and apigenin) were present in all cultivars, as well. However, one of the two identified querceitrin glucuronides (cluster 3) was detected only in cultivars Bounty, Florence, Jonsok, and, particularly, Polka (Fig. 4b). Taxifolin pentoside (cluster 15) was present in the samples grown in Finland (i.e. Bounty, Florence, Honeoye, Jonsok, Polka, Rumba, Salsa), and in cultivar Sonata grown in Estonia, with the highest level in Jonsok (Fig. 4c). It must be noted that thawing in microwave oven may affect the phenolic profile of strawberries: while the contents of non-anthocyanin strawberry phenolics may be either decreased or increased during microwave oven treatment depending on compound class, anthocyanins tend to decrease (Holzwarth, Korhummel, Carle, & Kammerer, 2012).

Although many clusters contained at least one terpene as a major component, clusters 5, 12 and 15 were distinguished for containing several terpene compounds. For example, a triterpene derivative (MW 486.3354) in cluster 5, present only in Florence, Capri, Dely, and Sonata, showed highest abundance in cultivar Florence. A triterpene hexoside in cluster 12 (MW 680.3786) also demonstrated very limited distribution, being present in only the Estonian-grown cultivars Capri, Clery, Dely, Ischia, Joly, and Linosa (Fig. 4d) with the highest abundance in cultivar Dely. Several sesquiterpene derivatives were detected in cluster 15 (Table S1).

3.4. Cultivar Jonsok contained particularly high levels of potential aroma and flavor precursors

In the KMC analysis, molecular features that were most abundant in Jonsok mainly belonged to cluster 1, which was the largest cluster representing 258 molecular features out of the total 1569 (16%) (Fig. 3). Butyrate, furans, acetophenone glycosides, and free and glycosylid forms of carboxylic and dicarboxylic acids were especially high in Jonsok. These types of metabolites are typical aroma and flavor precursors of fruits and berries (Beekwilder et al., 2004; Mayorga, Duque, Knapp, & Winterhalter, 2002; Tohge et al., 2014; Zabetakis & Holden, 1997). Jonsok has a characteristic taste being intensely sour and bitter with relatively low sweetness (Kärlund et al., 2015; Rosenfeld & Nes, 2000), and these properties may well be associated with the high levels of aromatic and aliphatic carboxylic acids, esters, ketones, and furanoles detected in this study (Kärlund et al., 2015; Perez, Ollas, Luaces, & Sanz, 2002; Zabetakis & Holden, 1997). In addition, the particularly high level of a procyanidin tetramer (cluster 1; Fig. S1) in Jonsok may further increase the bitterness of this cultivar (Soares et al., 2013).

In the very beginning of the domestication and breeding of Fragaria, strawberries with pleasant sensory characteristics were favored (Ulrich & Olbricht, 2013). Since then, the strawberry market has grown and new environmental challenges and consumer demands have emerged (Bestfleisch et al., 2014). Jonsok, launched in 1969 in Norway (Rosenfeld & Nes, 2000), is a relatively old strawberry cultivar bred to meet the requirements of commercial strawberry farming in northern areas, in respect of disease resistance (Koehler et al., 2012), productivity, and cold tolerance (Laugale & Lepse, 2007). Despite the well appreciated cultivation qualities, its sensory traits (sourness and bitterness) can make Jonsok less attractive to today's food industry and consumers.

3.5. Relatively high concentrations of several pelargonidin derivatives were detected in cultivar Polka

The levels of anthocyanin derivatives varied significantly between strawberry cultivars. Polka had the highest content for most of the identified pelargonidin derivatives, whereas cultivar Bounty had the highest content of a putative cyanidin glycoside derivative, the other main anthocyanin component in strawberries.

Anthocyanins have many putative health-promoting effects (Del Rio et al., 2013; Carrieri et al., 2013). The supply of anthocyanins from the every-day diet is usually good, even though they are absorbed from the gastrointestinal tract with varying efficiencies (Del Rio et al., 2013). In addition, anthocyanins are rather sensitive to the typical steps of strawberry processing in food production (Kärlund, Moor, Sandell, & Karjalainen, 2014b). Hence, the relatively higher anthocyanin content of Polka-based raw material could mitigate against processing-induced anthocyanin losses. Furthermore, the chemical composition of Polka is known to be relatively stable under different cultivation conditions (Kärlund et al., 2015), and this may further ease the quality control and standardization of strawberry products. The fact that cyanidin has demonstrated higher antioxidant capacity in comparison to pelargonidin (Wang, Cao, & Prior, 1997) may increase the breeding value of Bounty, which had the highest level of the cyanidin derivative.
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The present work highlights the complexity of fruit secondary metabolite profiles in different strawberry cultivars, which can distinguish between cultivars and suggest their genetic inter–relationships. The diversity of polyphenol components makes strawberries good sources of potentially bioactive constituents. However, the metabolomic approach also highlighted the presence of many other phytochemicals (including unknown components) which may also contribute to the physiology, quality and potential bioactivities of strawberries. We conclude that careful cultivar selection is essential to the breeding of modern strawberry cultivars with optimal and stable phytochemical compositions contributing to the potential functionalities and sensory qualities. Because the constancy of strawberries’ chemical composition in different farming and processing conditions is also cultivar–dependent it would be useful to survey the stability of the chemical composition of strawberry genotypes cultivated and processed with different methods.

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