JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

# Diversity of Phenolic Profiles in the Fruit Skin of *Prunus domestica* Plums and Related Species

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Supporting Information

**ABSTRACT:** The fruits of the European plum *Prunus domestica* exhibit a great diversity in appearance including skin colors. This study attempts to elucidate the phenylpropanoid and flavonoid profiles of 28 plum varieties belonging to *P. domestica* and related species as well as hybrids. A total of 49 phenolic compounds extracted from the fruit skin were quantitatively evaluated in an HPLC-DAD-based metabolomic study. The total phenolic contents of the cultivars varied among 0.4−29.9 mg/g fresh weight. The predominant anthocyanins were glycosides of cyanidin and peonidin, and rutin was the principal flavonol, whereas neochlorogenic acid and *n*-chlorogenic acid were the main hydroxycinnamic acids. Aside from these major phenolic classes, a group of tentatively identified flavones and several acylated flavonoids were also found. Principal component analysis revealed that anthocyanins and hydroxycinnamic acids contributed most to variety separation. The heterogeneity between the different varieties was also assessed using hierarchical cluster analysis of sample phenolics profile. A simple separation of species could not be found confirming the close relationship among them.

**KEYWORDS:** *phenylpropanoid, flavonoid, Rosaceae, plum, metabolome, chemotaxonomy*

## INTRODUCTION

The European plum, *Prunus domestica*, is commercially cultivated mainly in Germany, the United States, Romania, Bulgaria, and Serbia.[1] Its fruits exhibit a great diversity in size, shape, taste, and appearance. The fruits' skin colors, ranging from green over yellow to red and various blue tones, indicate strong differences in the biosynthesis and accumulation of the anthocyanin pigments. Because their biosynthesis is closely related to phenylpropanoids and other flavonoids, the colorless compounds may also show a wide quantitative diversity. Phenolic compounds are known to contribute to the health benefits of fruits, and particularly red, anthocyanin-rich fruits are regarded as antioxidant favorites. Besides the pigments, the noncolored phenolic compounds may also contribute to the fruits' beneficial effects.[2−4]

Studies have revealed the occurrence of several derivatives of hydroxycinnamic acids, flavonols, flavan-3-ols, and anthocyanins in the fruit skin of plums belonging to the species *Prunus domestica* and *Prunus salicina* (Table 1). Their qualitative phenolic profiles appeared very similar, except for the peonidin glycosides, which have not been detected in *P. salicina*. *P. domestica* seems to form a broader pattern of hydroxycinnamic acids and flavonols. However, the existing studies cover only a limited set of varieties, hindering a final conclusion regarding particular phenolic profiles of these species. The hybrid nature of the hexaploid species *P. domestica* complicates the situation. It is generally assumed that the cherry plum, *Prunus cerasifera*, and the sloe, *Prunus spinosa*, are ancestors of the European plum, but also *P. salicina* may be involved (reviewed by Neumüller[1]).

The immense structural diversity among phenolics makes them suitable as chemotaxonomic markers. Phenolic fingerprints have successfully been used for the characterization and identification of varieties from sweet and sour cherries,[5,6] pelargonium,[7] roses,[8] azalea,[9] and, more recently, *Gynostemma pentaphyllum*.[10] Using phenolic profiles together with enzyme patterns of the bark of *P. domestica* trees, Groh et al.[11] were able to distinguish between 12 cultivars. In a chemometric study, Nunes et al.[12] attempted the identification of variety and even geographical origin of plums with promising results.

The aim of our study was to gain knowledge about the variability of phenolic profiles of the fruit skin within the species *P. domestica*. For this purpose 27 cultivars and clones from our breeding program were analyzed including interspecific hybrids with *P. cerasifera* and *P. spinosa* together with fruits from their parents. One *P. salicina* variety was also included. It was the goal to elucidate to what extent the nonvisible phenolic compounds vary in the skin of plums to get some insight into the possible biosynthetic pathways and eventually identify qualitative differences among the varieties or quantitative affinities among closely related progeny. Varieties were classified and compared against one another using chemometric analysis, including principal component analysis (PCA) and hierarchical cluster analysis, and the intraspecific heterogeneity was assessed.

Received: August 23, 2012
Revised: November 6, 2012
Accepted: November 10, 2012
Published: November 10, 2012

**Table 1. Phenolic Pattern of Plums Belonging to *P. domestica* and *P. salicina* As Described in the Literature**

| phenolic compound | *P. domestica* | *P. salicina* | references |
|---|---|---|---|
| 3-*O*-caffeoylquinic acid (neochlorogenic acid) | x | x | Möller and Herrmann, 1983; Donovan et al., 1998; Chun et al., 2004; Nunes et al., 2008; Slimestad et al., 2009 |
| 3-*O*-*p*-coumaroylquinic acid | x | | Möller and Herrmann, 1983; Slimestad et al., 2009 |
| 3-*O*-*p*-feruloylquinic acid | x | | Möller and Herrmann, 1983 |
| 4-*O*-caffeoylquinic acid (cryptochlorogenic acid) | x | x | Möller and Herrmann, 1983; Tomás-Barberán et al., 2001; Slimestad et al., 2009 |
| 4-*O*-*p*-coumaroylquinic acid | x | | Möller and Herrmann, 1983 |
| 5-*O*-caffeoylquinic acid (*n*-chlorogenic acid) | x | x | Möller and Herrmann, 1983; Donovan et al., 1998; Tomás-Barberán et al., 2001; Chun et al., 2003; Nunes et al., 2008; Slimestad et al., 2009 |
| 5-*O*-*p*-coumaroylquinic acid | x | | Möller and Herrmann, 1983 |
| 5-*O*-*p*-feruloylquinic acid | x | | Möller and Herrmann, 1983 |
| caffeic acid | x | | Donovan et al., 1998 |
| caffeoylshikimic acid | x | x | Nunes et al., 2008 |
| *p*-coumaric acid | x | | Donovan et al., 1998 |
| feruloylquinic acid | x | | Nunes et al., 2008 |
| quercetin 3-glucoside (isoquercitrin) | x | x | Tomás-Barberán et al., 2001; Nunes et al., 2008; Chun et al., 2009; Slimestad et al., 2009 |
| quercetin 3-rutinoside (rutin) | x | x | Donovan et al., 1998; Tomás-Barberán et al., 2001; Chun et al., 2008; Nunes et al., 2008; Slimestad et al., 2009 |
| quercetin 3-galactoside (hyperoside) | x | x | Nunes et al., 2008; Chun et al., 2010 |
| quercetin 3-rhamnoside | | x | Tomás-Barberán et al., 2001; Nunes et al., 2008 |
| isorhamnetin-rutinoside | x | | Nunes et al., 2008 |
| myricetin | x | | Nunes et al., 2008 |
| cyanidin 3-glucoside | x | x | Tomás-Barberán et al., 2001; Chun et al., 2006; Usenik et al., 2008; Slimestad et al., 2009 |
| cyanidin 3-rutinoside | x | x | Tomás-Barberán et al., 2001; Chun et al., 2005; Usenik et al., 2008; Slimestad et al., 2009 |
| peonidin 3-glucoside | x | | Chun et al., 2007; Usenik et al., 2008; Slimestad et al., 2009 |
| peonidin 3-rutinoside | x | | Usenik et al., 2008; Slimestad et al., 2009 |
| catechin | x | x | Donovan et al., 1998; Nunes et al., 2008 |
| epicatechin | x | x | Nunes et al., 2008 |
| procyanidin B1 | x | x | Tomás-Barberán et al., 2001; Nunes et al., 2008 |
| procyanidin B2 | x | x | Tomás-Barberán et al., 2001; Nunes et al., 2008 |
| procyanidin B4 | | x | Tomás-Barberán et al., 2001 |
| procyanidin B7 | x | | Nunes et al., 2008 |

## MATERIAL AND METHODS

**Plant Material.** Fruits from 28 varieties (Table 2) were harvested fully ripe (good eating quality, defined by sweetness, taste, and softness according to each variety's characteristics) during the season (from August to October) in 2010 at the experimental farm of the Center of Life Sciences, Weihenstephan, in Freising, Germany. The skin of fruits was quickly removed, shock frozen in liquid nitrogen, and stored at −20 °C for further analysis. For each variety three samples each consisting of three fruits were analyzed separately.

**Chemicals.** All solvents used for HPLC were of gradient grade quality, and those used for extraction were of analytical grade quality. Naturstoff-Reagenz A was purchased from Roth, sulfatase enzyme from Sigma-Aldrich, and dimethylaminocinnamaldehyde from Merck. The phenolic standards were obtained from Polyphenols Laboratories AS, Sandnes, Norway (cyanidin 3-glucoside, cyanidin 3-rutinoside, peonidin 3-glucoside, and peonidin 3-rutinoside), from Roth, Karlsruhe, Germany (*n*-chlorogenic acid, caffeic acid, catechin, and epicatechin), and from Extrasynthese, Genay, France (hyperin, isoquercitrin, rutin, avicularin, and isorhamnetin-3-glucoside). Neo- and cryptochlorogenic acid (3′- and 4′-caffeoylquinic acids, respectively) were prepared by isomerization of *n*-chlorogenic acid (5′-caffeoylquinic acid) in a phosphate buffer solution.[13]

**Extraction of Phenolic Compounds.** The frozen skin was ground with liquid nitrogen and quartz sand using a mortar and a pestle. The solvent ($HCO_2H$/MeOH, 5:95, v/v) containing 0.04 mg/mL flavone as internal standard was added, and extraction was performed in an ultrasonic bath at 4 °C for 30 min. After centrifugation (1120*g*, 10 min, 4 °C), the solvent of the supernatant was removed in a rotation vacuum evaporator. The dried samples were kept at −20 °C until further analysis. The dried residues were resuspended in $HCO_2H$/MeOH (5:95, v/v) to a concentration of 1.25 g of fresh material/mL solvent. Aliquots (7 μL) were injected into the HPLC system.

**HPLC Analysis.** The HPLC system consisted of two pumps (model 422, Kontron Instruments, Germany), an automatic sample injector (model 231, Gilson Abimed Systems, Germany), and a photodiode array detector (Kontron 540, Kontron Instruments). For post column derivatization[14,15] a further Gynkotek analytical HPLC pump (model 300C, Germering, Germany) and a visible detector (Kontron Detector 432, Kontron Instruments) were set at 640 nm for selective detection of catechins and proanthocyanidins. Phenolic compounds were separated using a Kinetex 2.6 μm PFP column (150 × 4.6 mm, 100A, Phenomenex) and eluted with 5% HCOOH (solvent A) and HPLC grade MeOH (solvent B) at a flow rate of 0.5 mL/min. The gradient profile was as follows: 0% B, 5 min; 0−2.5% B, 2.5 min; 2.5% B, 5 min; 2.5−5% B, 12.5 min; 5% B, 5 min; 5−10% B, 20 min; 10−20% B, 40 min; 20−30% B, 40 min; 30−40% B, 10 min; 40−50% B, 10 min; 50−90% B, 10 min; 90% B, 15 min. The software Geminyx-III was applied for the integration and quantification of phenolic compounds. For quantification, phenolic compounds were grouped into seven categories and monitored at related wavelengths: 280 nm (unknown phenolic compounds), 320 nm (hydroxycinnamic acids, flavones, and acylated flavonoids), 350 nm (flavonols), 540 nm (anthocyanins), and 640 nm (procyanidins and flavan-3-ols). The calculation was done on the basis of specific response factors for reference compounds. Unknown phenolic compounds, hydroxycinnamic acids, and acylated flavonoids were calculated as chlorogenic acid, flavones as apigenin, flavonols as rutin, anthocyanins as cyanidin 3-glucoside, and monomeric flavan-3-ols as catechin and epicatechin, respectively.

**Thin Layer Chromatography.** Cellulose plates (20 × 10 mm) were used as the stationary phase. Plates were developed with the mobile phase *n*-butyl alcohol/acetic acid/water (40:10:22, v/v/v). For visualization of phenolic compounds UV light was used and spraying with Naturstoff-Reagenz A.

**Table 2. Varieties Used for the Discrimination Study and Their Genetic Origin**

| variety | origin | skin color | species |
|---|---|---|---|
| Cacaks Fruchtbare = Cacanska rodna | Stanley × Pozegaca | dark blue | *P. domestica* |
| Große Grüne Reneklode = Green Gage | traditional variety | green | *P. domestica* |
| Hoh 6087 | Jojo × Hanita | reddish blue | *P. domestica* |
| Jojo | Ortenauer × Stanley | dark blue, violet | *P. domestica* |
| Katinka | Ortenauer × Ruth Gerstetter | dark violet, blue | *P. domestica* |
| Krichele Dürnau | wild type | blue | *P. domestica* |
| Miragrande | Herrenhäuser Mirabelle × gelbe Zwetsche | yellow | *P. domestica* |
| Ortenauer | traditional variety | dark blue | *P. domestica* |
| Pogauner | traditional variety | reddish blue | *P. domestica* |
| President | traditional variety | dark violet−reddish | *P. domestica* |
| Topper | Cacaks Beste × Auerbacher | reddish blue | *P. domestica* |
| Toptaste | Valor × Hauszwetsche | dark blue | *P. domestica* |
| Haferschlehe | wild type | blue | *P. spinosa* |
| Späte Myrobalane | wild type | yellow | *P. cerasifera* |
| Tatjana | | yellow-red | *P. cerasifera*/*P. salicina* |
| Songold | | yellow | *P. salicina* |
| Wei 252 | Jojo × *P. cerasifera* | blue | *P. domestica* × *P. cerasifera* |
| Wei 261 | Jojo × *P. cerasifera* | dark blue | *P. domestica* × *P. cerasifera* |
| Wei 256 | Jojo × *P. cerasifera* | dark blue | *P. domestica* × *P. cerasifera* |
| Wei 266 | Jojo × *P. cerasifera* | reddish blue | *P. domestica* × *P. cerasifera* |
| Wei 267 | Jojo × *P. cerasifera* | reddish | *P. domestica* × *P. cerasifera* |
| Wei 238 | Jojo × *P. spinosa* | dark blue | *P. domestica* × *P. spinosa* |
| Wei 243 | Jojo × *P. spinosa* | dark blue | *P. domestica* × *P. spinosa* |
| Wei 244 | Jojo × *P. spinosa* | dark blue | *P. domestica* × *P. spinosa* |
| Wei 247 | Jojo × *P. spinosa* | reddish blue | *P. domestica* × *P. spinosa* |
| Wei 1660 | Jojo × *P. spinosa* | dark blue | *P. domestica* × *P.spinosa* |
| Prunus Fruticanus Slaponice | | dark blue | *P. domestica* × *P. spinosa* |
| Aprimira | Herrenhäuser Mirabelle × Orangered ? | yellow-red | *P. domestica* × *P. armeniaca* ? |

**Acid Hydrolysis.** Twenty microliters of the purified fraction diluted with 80 μL of MeOH was transferred into a test tube, and 100 μL of 1 M HCl was added. The mixture was incubated for 10 min in a boiling water bath.

**Enzymatic Hydrolysis.** Thirty microliters of the purified fraction was mixed with a buffer/enzyme solution in a test tube. The preparation of the buffer/enzyme solution was made by adding 2 mg of sulfatase enzyme into 300 μL of 0.1 M sodium acetate buffer, pH 4.6. Samples were incubated in a water bath at 37 °C for 16 h. After incubation, methanol was added to stop the enzyme. The supernatant containing the aglycone was extracted and placed into a new tube. The pellet was washed three times with 300 μL of ethyl acetate. After every wash, the samples were centrifuged for 1 min (4 °C, 1120*g*).

**Principal Component Analysis (PCA) and Hierarchical Cluster Analysis.** Principal component and hierarchical cluster analyses were used for the differentiation and classification of plum varieties. Phenolics profiles of 28 plum skin samples were assessed to test for possible heterogeneity among different varieties and species. Both methods are unsupervised clustering methods requiring no knowledge of the data set and act to reduce the dimensionality of multivariate data while preserving most of the variance within.[16] This method allows the clustering of the samples according to intrinsic variance. In this study, PCA and hierarchical cluster analysis were performed using three R packages, PCA methods, Heatplus, and gplots, which can be downloaded freely as an R package from the Metlin Metabolite Database (http://137.131.20.83/download/) under the R 2.9.2 environment.

## RESULTS AND DISCUSSION

**Identification of Phenolic Compounds.** Around 100 peaks were detected via HPLC analyses of all studied varieties. Among them, 49 compounds were selected for the discrimination study (Table 3). Minor components detected in only a few samples were ignored. For identification and characterization of the phenolic compounds, an extract from the variety Jojo was fractionated during several HPLC runs to purify the main components. These fractions were further characterized by TLC and hydrolyzed to obtain the respective aglycones. These results were compared with authentic standards; for the anthocyanins, cyanidin 3-glucoside, cyanidin 3-rutinoside, peonidin 3-glucoside, and peonidin 3-rutinoside; for the flavonols, hyperin, isoquercitrin, rutin, avicularin,and isorhamnetin-3-glucoside; for the hydroxycinnamic acids, *cis*-neochlorogenic acid, *trans*-neochlorogenic acid, *trans*-cryptochlorogenic acid, and *trans*-*n*-chlorogenic acid. The remaining compounds were classified according to their UV−visible absorbance characteristics to anthocyanins, flavonols, and hydroxycinnamic acids. Another group of compounds showed similar absorbance behavior as the flavone apigenin. These were therefore tentatively classified as flavones, which have not been described in plums so far. A further group of components exhibit UV absorbance spectra very similar to caffeic acid but were rather lipophilic. This is typical for acylated flavonoids, which may consist of a glycosylated flavon(ol) moiety with a hydroxycinnamic acid attached as previously described in ref 17. The identity of flavan-3-ols catechin and epicatechin was confirmed by postcolumn derivatization with dimethylaminocinnamaldehyde.[14]

The phenolic profiles were in general agreement with previous reports, revealing cyanidin-3-glucoside, cyanidin-3-rutinoside, peonidin-3-glucoside, and peonidin-3-rutinoside as predominant anthocyanins,[18,19] rutin as the principal flavonol,[20] and neochlorogenic acid and chlorogenic acid as the main hydroxycinnamic acids.[21,22] The total phenolic contents in the skin of plum varieties showed large differences (0.4−29.9 mg/g FW), among which 'Prunus Fruticanus Slaponice' exhibited the highest level

**Table 3. Phenolic Compounds from Plum Skins Separated by HPLC and Used for Discrimination Studies**

| compound name/ acronym | retention time (min) | compound name/ acronym | retention time (min) |
|---|---|---|---|
| *cis*-neochlorogenic acid | 15.0 | flavone 7 | 110.0 |
| flavone 1 | 15.4 | peonidin-3-glu | 107.3 |
| *trans*-neochlorogenic acid | 20.9 | peonidin-3-rut | 115.3 |
| *p*-coumaric acid deriv | 28.0 | quercetin 3-galactoside | 128.5 |
| *trans*-cryptochlorogenic acid | 30.0 | quercetin-glyc 1 | 131.5 |
| catechin | 35.7 | quercetin 3-glucoside | 132.6 |
| HCA 6 | 46.1 | quercetin 3-rutinoside | 133.3 |
| HCA 7 | 47.5 | flavonol 6 | 134.8 |
| *trans-n*-chlorogenic acid | 49.2 | quercetin 3-arabinoside (avicularin) | 135.4 |
| HCA 9 | 56.8 | acyl 3 | 137.1 |
| HCA 10 | 57.6 | acyl 4 | 138.8 |
| flavone 2 | 58.6 | acyl 5 | 140.6 |
| epicatechin | 63.1 | antho 26 | 140.8 |
| flavone 3 | 63.7 | quercetin-gly 2 | 141.0 |
| flavone 4 | 65.6 | flavonol 8 | 142.4 |
| flavone 5 | 66.2 | flavone 9 | 143.9 |
| HCA 11 | 71.1 | isorhamnetin-gly | 145.6 |
| antho 6 | 72.8 | isorhamnetin-3-glu | 147.2 |
| HCA 12 | 75.9 | acyl 6 | 148.6 |
| cyanidin-3-glu | 82.5 | acyl 9 | 150.3 |
| cyanidin-3-rut | 92.9 | acyl 13 | 153.4 |
| antho 16 | 99.2 | acyl 14 | 153.7 |
| flavone 6 | 99.8 | acyl 18 | 154.0 |
| antho 17 | 103.1 | acyl 19 | 154.5 |
| antho 18 | 105.6 | | |

followed by 'Toptaste', 'Krichele Dürnau', and 'Wei 238' (Figure 1). The *P. cerasifera* variety 'Späte Myrobalane' had the lowest total phenolic content. The hybrids of *P. domestica* × *P. cerasifera* ('Wei 266', 'Wei 256', 'Wei 267', 'Wei 252', and 'Wei 261') have lower phenolic contents than the average level, whereas the hybrids of *P. domestica* × *P. spinosa* have both high and low phenolic content varieties. Compared with previous data on plum flesh,[22] the concentration of phenolic compounds in skins was much higher.

Anthocyanins were detected in most samples (Figure 2; Table 4; Supporting Information, Table 1S) except for the yellow and green varieties 'Späte Myrobalane' and 'Große Grüne Reneklode' (= 'Green Gage'). The ratio of total anthocyanins/total phenolics was >50% for most of the plum varieties. Albeit, for some yellow and green varieties the total anthocyanins/total phenolics ratio was much lower, that is, 'Aprimira' (0.2%), 'Songold' (0.3%), and 'Miragrande' (1%). The range of total anthocyanins in the 28 plum varieties was 0.01−22.3 mg/g FW with an average concentration of 6.5 mg/g FW. The dark blue 'Prunus Fruticanus Slaponice' had the highest anthocyanin content, whereas 'Aprimira' showed the lowest amounts. The anthocyanins cyanidin 3-glucoside, cyanidin 3-rutinoside, peonidin 3-glucoside, and peonidin 3-rutinoside accounted for 99% of total anthocyanins in the skins of all plum varieties. Cyanidin 3-glucoside and cyanidin 3-rutinoside were most frequent in plum varieties, whereas peonidin glycosides were found to occur irregularly, which was also reported for Norwegian plums.[18]

Total flavonol contents were found in the range of 0.2−3.4 mg/g FW with an average value of 1.1 mg/g FW (Table 5; Supporting Information, Table 2S). Samples of 'Wei 247', 'President', and 'Tatjana' showed the highest flavonol content, whereas 'Späte Myrobalane' had the lowest amounts. The ratio of total flavonol/total phenolics was <30% in most of the varieties, except for 'Späte Myrobalane' (35%) and 'Tatjana' (63%). The level of flavonols was lower than that of anthocyanins in most varieties. Among flavonols, rutin accounted for ∼50% of the total flavonols in agreement with a previous study.[20] The second most abundant flavonol was a quercetin derivative (quercetin glycoside 2, retention time 141 min), which accounted for 16% of total flavonols. Quercetin 3-glucoside (isoquercitrin) and isorhamnetin 3-glucoside, together with quercetin 3-rutinoside (rutin), were found in all of the samples with concentration ranges of 0.01−0.25, 0.01−0.26, and 0.05−1.3 mg/g, respectively. In contrast, quercetin 3-arabinoside (avicularin) was detected at high levels exclusively in 'Krichele Dürnau' and 'Prunus Fruticanus Slaponice'. It should be noted that flavonols showed qualitative differences across plum samples.

Hydroxycinnamic acids (HCA) are mainly found as chlorogenic acids with neochlorogenic acid as the major phenolic acid in most varieties. The range of total HCA varied from 0.03 to 12.2 mg/g FW with an average content of 3.6 mg/g FW (Table 6; Supporting Information, Table 3S). Highest levels of HCAs were found in 'Toptaste' (>3 times than the mean value), whereas 'Späte Myrobalane' (*P. cerasifera*) had again the lowest amounts. The total HCAs content of hybrids of *P. domestica* × *P. cerasifera* ('Wei 252', 'Wei 261', 'Wei 266', 'Wei 256', and 'Wei 267') was <1 mg/g FW, whereas *P. domestica* × *P. spinosa* hybrids ('Wei 238' and 'Wei 247') had high levels. The ratio of total HCAs/total phenolics showed large variation from 3 to 88%, with a mean value of 31%. Varieties 'Große Grüne Reneklode' (88%), 'Miragrande' (84%), and 'Aprimira' (81%), which showed low anthocyanin contents, had >80% HCAs of total phenolics. With respect to concentration levels (Table 6), neochlorogenic acid was found most abundant, accounting for 84% of total HCAs, whereas chlorogenic acid accounted for 10%. Cryptochlorogenic acid was found exclusively in four samples including 'Hoh 6087', 'Miragrande', 'Toptaste', and 'Topper' belonging to *P. domestica*. Chlorogenic acid was common in all samples, whereas neochlorogenic acid was absent in 'Späte Myrobalane'. Among Norwegian plums derived from *P. domestica*, neochlorogenic acid was also found to be the major phenolic compound, which varied significantly among varieties.[18]

Flavones presented a minor class in the skin of plums with $\sim^1/_{200}$ of mean values for anthocyanins and $\sim^1/_{100}$ for HCAs (Supporting Information, Table 4S). Except for 'Späte Myrobalane' and 'Aprimira', flavones were found in all other varieties, with a concentration ranging from 0.2 to 10.2 mg/100 g FW. Varieties 'Wei 247', 'Wei 243', 'Wei 238', 'Krichele Dürnau', and 'Toptaste' were most enriched in flavones.

Acylated flavonoids are dimers consisting of at least one flavonoid moiety and at least one HCA moiety, of common occurrence in plums. The concentration of acylated flavonoids in this study ranged from 1.5 to 30 mg/100 g FW with a mean value of 11 mg/100 g FW (Supporting Information, Table 5S). Samples 'Wei 238' and 'Toptaste' had the highest levels of acylated flavonoids, whereas 'Prunus Fruticanus Slaponice' was the least enriched in that class of phenolics.

The flavan-3-ols detected in this study were mainly catechin and epicatechin. It was found that for most of the plum varieties, procyanidin peaks were not significant, except for 'Songold', the only variety belonging to *P. salicina* in this study. The range of total flavan-3-ols in plum varieties was 0.5−43.2 mg/100 g FW,

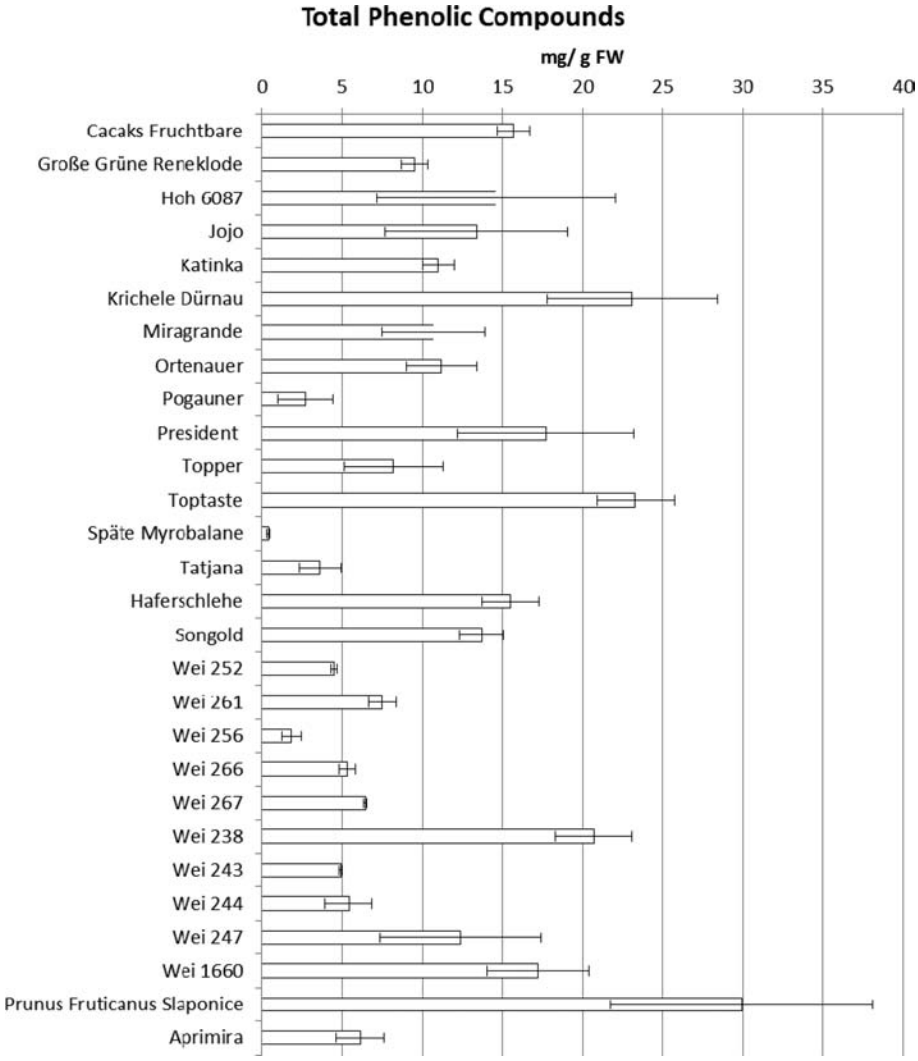


**Figure 1.** Total phenolic contents (mg/g fresh weight) in the skin of the plum varieties and clones (mean of three replicates ± standard deviation).

except for 'Songold' with a concentration of 485.2 mg/100 g FW mostly due to catechin (Table 7). The presence of catechin and epicatechin in our plums grown in Germany is not in accordance with reports for Norwegian plums,[18] and whether these phenolics could serve as markers for plum origin has to be further studied. In the varieties 'Späte Myrobalane', 'Wei 266', 'Wei 256', 'Wei 267', 'Wei 252', 'Wei 247', and 'Wei 244' no flavan-3-ols could be detected.

*Principal Component Analysis and Hierarchical Cluster Analysis.* Although different phenolic profiles were observed by simple visual inspection of the HPLC chromatograms of the different varieties, we attempted to analyze the plum data set in a more holistic way using PCA to explore the relative variability within the different varieties. PCA is an unsupervised clustering method requiring no knowledge of the data set and acts to reduce the dimensionality of multivariate data while preserving most of the variance within the data.[15] Considering 50 variables as analytical data (complete phenolics profile), PCA was able to discriminate among varieties. Four principal components (PCs) were required to capture almost 99% of the variance. The main principal component (PC) to differentiate between samples, that is, PC1, accounts for 59% of plum variance (Supporting Information, Figure 1S, A). The PC1/PC2 scores plot shows that on the right side of the plot, samples for 'Prunus Fruticanus Slaponice', 'Krichele Dürnau', 'Wei 1660', 'Wei 238', 'Hoh 6087', 'Toptaste', and 'Jojo' are positioned (positive PC1 values), whereas on the far left side, most other samples are located (negative PC1 values). Examination of the loadings plot suggested that the variables referred to cyanidin 3-glucoside, cyanidin 3-rutinoside, peonidin 3-glucoside, and peonidin 3-rutinoside contributing for discrimination of species. PC2 explained 32% of the variation captured between samples and was related to chlorogenic acid and neochlorogenic acid, both contributing negatively to PC2 (Supporting Information, Figure 1S, B). Neochlorogenic acid contributed the most in species discrimination.

Cluster analysis of the different plum samples was used as an additional exploratory tool to assess the heterogeneity between different varieties. Hierarchical cluster analysis showed two clear major clusters, of 6 and 22 genotypes (Figure 3), referred to as groups 1A and 1B, respectively. Inspection of the dendrogram showed that samples 'Prunus Fruticanus Slaponice', 'Krichele Dürnau', 'Wei 1660', 'Wei 238', 'Hoh 6087', and 'Jojo' were the most distant in comparison to other samples and were grouped in 1A. Inspection of cluster 1A, for metabolites mediating for the clustering pattern, showed that individuals from cluster 1A were enriched in phenolic compounds, mainly cyanidin 3-glucoside, cyanidin 3-rutinoside, peonidin 3-glucoside, and

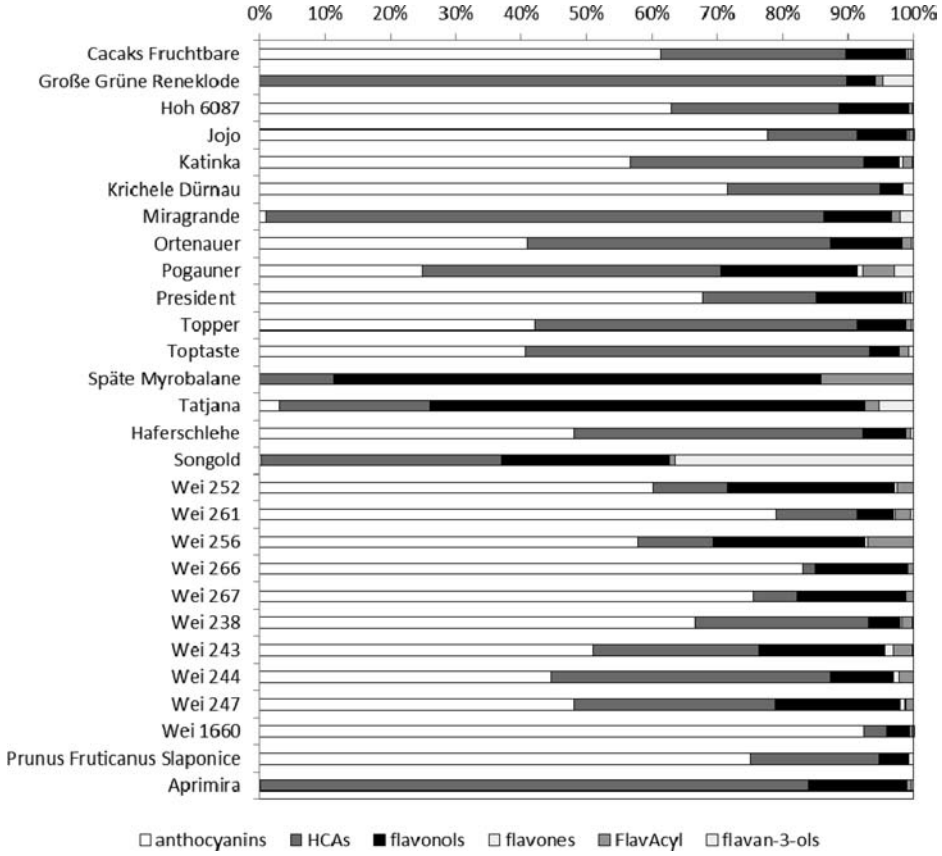


**Figure 2.** Qualitative profiles of phenolic classes in the fruit skin of plum varieties and clones. HCA, hydroxycinnamic acids; FlavAcyl, acylated flavonoids.

**Table 4. Anthocyanin Concentrations in the Fruit Skins of Plum Varieties and Clones**[a]

| | cya-3-glu | cya-3-rut | peo-3-glu | peo-3-rut |
|---|---|---|---|---|
| Cac. Fruchtbare | 190.0 ± 63.3 | 487.5 ± 70.5 | 29.9 ± 8.1 | 238.6 ± 56.6 |
| G. G. Reneklode | nd | nd | nd | nd |
| Hoh 6087 | 52.2 ± 36.7 | 318.6 ± 179.4 | 17.4 ± 13.4 | 215.5 ± 200.9 |
| Jojo | 57.1 ± 34.1 | 363.1 ± 168.9 | 32.1 ± 22.6 | 564.4 ± 333.6 |
| Katinka | 94.6 ± 3.8 | 266.7 ± 13.4 | 24.9 ± 2.1 | 224.0 ± 15.4 |
| Krichele D. | 163.3 ± 27.6 | 487.0 ± 93.6 | 158.9 ± 22.9 | 813.5 ± 165.0 |
| Miragrande | 2.4 ± 3.5 | 2.9 ± 3.2 | nd | nd |
| Ortenauer | 47.0 ± 16.7 | 239.9 ± 80.3 | 10.0 ± 3.8 | 148.9 ± 33.8 |
| Pogauner | 3.7 ± 4.0 | 31.4 ± 29.1 | nd | 27.2 ± 17.1 |
| President | 274.2 ± 111.8 | 793.4 ± 224.9 | 13.5 ± 4.8 | 85.8 ± 17.6 |
| Topper | 18.7 ± 13.4 | 125.8 ± 74.5 | 8.5 ± 7.4 | 184.6 ± 97.9 |
| Toptaste | 182.1 ± 78.1 | 620.6 ± 34.4 | 11.6 ± 3.6 | 113.4 ± 36.1 |
| Sp. Myrobalane | nd | nd | nd | nd |
| Tatjana | 1.1 ± 1.8 | 7.9 ± 10.8 | nd | 1.0 ± 1.7 |
| Haferschlehe | 119.9 ± 24.6 | 363.0 ± 30.6 | 83.5 ± 19.1 | 156.4 ± 14.1 |
| Songold | 0.8 ± 1.0 | 3.3 ± 3.5 | nd | nd |
| Wei 252 | 11.0 ± 2.0 | 66.7 ± 4.4 | 8.4 ± 0.9 | 168.7 ± 22.7 |
| Wei 261 | 34.4 ± 6.8 | 324.7 ± 14.2 | 9.6 ± 2.8 | 179.4 ± 22.3 |
| Wei 256 | 2.1 ± 1.2 | 9.8 ± 5.6 | 6.3 ± 2.3 | 77.0 ± 35.5 |
| Wei 266 | 27.9 ± 5.3 | 146.6 ± 8.2 | 15.5 ± 4.2 | 217.2 ± 52.3 |
| Wei 267 | 17.9 ± 3.8 | 153.3 ± 22.9 | 13.8 ± 4.0 | 261.6 ± 24.8 |
| Wei 238 | 171.8 ± 23.7 | 396.6 ± 48.7 | 134.5 ± 16.7 | 638.0 ± 140.4 |
| Wei 243 | 24.5 ± 5.7 | 176.3 ± 35.3 | 1.5 ± 0.1 | 35.0 ± 4.1 |
| Wei 244 | 22.6 ± 4.3 | 140.3 ± 32.3 | 6.0 ± 1.4 | 66.0 ± 15.1 |
| Wei 247 | 61.1 ± 28.6 | 165.0 ± 73.4 | 72.2 ± 36.6 | 282.7 ± 111.8 |
| Wei 1660 | 31.3 ± 5.0 | 314.1 ± 63.8 | 142.9 ± 48.0 | 971.3 ± 309.1 |
| Pr. Frutic. | 219.9 ± 68.5 | 490.9 ± 155.2 | 253.8 ± 60.9 | 1230.6 ± 397.7 |
| Aprimira | 0.5 ± 0.5 | 1.0 ± 1.1 | nd | nd |

[a]Milligrams per 100 g fresh weight (FW); mean of three replicates ± standard deviation; minor components are given in the Supporting Information, Table 1S.

**Table 5. Flavonol Concentrations in the Fruit Skins of Plum Varieties and Clones[a]**

| | rutin | hyperin | quercetin-glyc 1 | isoquercitrin | quercetin-glyc 2 | isorha-glyc | isorha 3-glu |
|---|---|---|---|---|---|---|---|
| Cac. Fruchtbare | 97.9 ± 14.5 | 5.6 ± 0.3 | 7.1 ± 1.6 | 6.8 ± 0.5 | nd | 5.3 ± 0.6 | 11.2 ± 1.8 |
| G. G. Reneklode | 20.1 ± 3.9 | 1.2 ± 0.1 | 3.1 ± 0.9 | 2.0 ± 0.5 | nd | 6.4 ± 2.2 | 6.5 ± 1.3 |
| Hoh 6087 | 109.3 ± 55.4 | nd | 4.7 ± 3.3 | 18.4 ± 11.0 | nd | 6.3 ± 3.7 | 8.6 ± 4.8 |
| Jojo | 66.1 ± 9.8 | 5.1 ± 2.1 | 10.7 ± 0.1 | 4.4 ± 1.1 | nd | 3.9 ± 1.4 | 4.3 ± 0.6 |
| Katinka | 18.7 ± 0.1 | 7.2 ± 0.1 | 2.6 ± 1.0 | 5.5 ± 0.2 | 4.9 ± 0.5 | 6.3 ± 0.4 | 8.2 ± 0.5 |
| Krichele D. | 29.7 ± 6.9 | 4.1 ± 1.4 | 4.8 ± 1.3 | 8.3 ± 1.8 | 18.0 ± 5.6 | 2.8 ± 0.7 | 2.3 ± 0.5 |
| Miragrande | 52.8 ± 25.3 | 9.6 ± 10.9 | 20.2 ± 15.9 | 5.2 ± 2.8 | 10.4 ± 7.8 | nd | 6.5 ± 3.9 |
| Ortenauer | 39.7 ± 19.1 | 10.1 ± 6.3 | 4.7 ± 2.9 | 3.4 ± 1.2 | 15.4 ± 8.0 | 11.7 ± 4.4 | 26.2 ± 7.4 |
| Pogauner | 11.8 ± 3.6 | 2.8 ± 1.1 | 1.9 ± 0.1 | 3.3 ± 1.1 | 6.4 ± 1.9 | 5.1 ± 1.2 | 14.6 ± 2.7 |
| President | 31.8 ± 62.5 | 28.9 ± 17.5 | 40.1 ± 23.9 | 18.4 ± 8.2 | nd | 7.9 ± 4.1 | 4.7 ± 1.7 |
| Topper | 40.0 ± 40.0 | 0.8 ± 1.1 | 4.8 ± 5.8 | 1.5 ± 1.7 | nd | 2.1 ± 2.8 | 5.3 ± 4.3 |
| Toptaste | 44.3 ± 4.7 | 19.5 ± 6.9 | 16.8 ± 4.0 | 8.9 ± 4.3 | nd | nd | 2.5 ± 0.7 |
| Sp. Myrobalane | 5.5 ± 2.7 | nd | nd | 1.0 ± 0.1 | 4.2 ± 0.3 | nd | 1.7 ± 0.9 |
| Tatjana | 108.1 ± 59.2 | 8.3 ± 5.9 | nd | 5.8 ± 6.1 | 53.9 ± 27.7 | nd | 17.8 ± 9.6 |
| Haferschlehe | 42.3 ± 3.9 | 2.9 ± 0.6 | 2.9 ± 1.1 | 11.9 ± 2.5 | 23.6 ± 2.3 | 5.6 ± 0.4 | 5.7 ± 0.2 |
| Songold | 119.4 ± 31.3 | nd | 1.2 ± 0.2 | 24.9 ± 11.3 | 97.3 ± 60.9 | nd | 18.9 ± 9.4 |
| Wei 252 | 40.0 ± 6.1 | 3.8 ± 1.0 | 5.5 ± 2.9 | 6.5 ± 1.1 | 28.4 ± 4.2 | 5.4 ± 0.5 | 15.1 ± 1.5 |
| Wei 261 | 26.7 ± 1.8 | 1.3 ± 0.5 | 2.3 ± 0.8 | 2.7 ± 0.4 | 6.3 ± 0.9 | 2.1 ± 0.3 | 0.6 ± 0.1 |
| Wei 256 | 24.8 ± 7.4 | nd | 1.0 ± 0.4 | 2.9 ± 0.8 | 4.5 ± 1.3 | 3.4 ± 1.0 | 2.4 ± 0.7 |
| Wei 266 | 37.4 ± 7.9 | 1.8 ± 0.1 | 4.6 ± 1.3 | 2.6 ± 0.2 | 12.9 ± 2.0 | 3.5 ± 0.7 | 2.3 ± 0.5 |
| Wei 267 | 62.3 ± 11.7 | 1.5 ± 0.4 | 5.6 ± 0.7 | 3.8 ± 0.2 | 17.2 ± 0.6 | 5.9 ± 0.5 | 3.0 ± 0.9 |
| Wei 238 | 31.4 ± 4.9 | 2.4 ± 0.7 | 0.8 ± 1.4 | 14.9 ± 1.8 | 27.1 ± 4.1 | 5.3 ± 1.3 | 7.2 ± 1.2 |
| Wei 243 | 29.1 ± 2.6 | 3.6 ± 0.3 | 4.3 ± 1.1 | 4.7 ± 1.7 | 20.6 ± 2.8 | 4.7 ± 0.7 | 17.6 ± 3.8 |
| Wei 244 | 15.8 ± 6.9 | 1.4 ± 0.9 | 2.3 ± 1.4 | 2.7 ± 1.4 | 12.4 ± 6.9 | 1.2 ± 0.7 | 4.2 ± 1.9 |
| Wei 247 | 65.1 ± 25.7 | 2.1 ± 1.1 | 6.7 ± 2.4 | 17.8 ± 7.1 | 60.2 ± 23.6 | 10.5 ± 3.8 | 25.9 ± 8.4 |
| Wei 1660 | 26.1 ± 2.8 | 7.5 ± 0.2 | 4.1 ± 0.3 | 2.4 ± 0.4 | 5.9 ± 1.2 | 2.8 ± 0.9 | 5.4 ± 2.3 |
| Pr. Frutic. | 26.6 ± 6.4 | nd | nd | 12.0 ± 2.9 | 47.4 ± 11.8 | 4.1 ± 1.0 | 3.6 ± 1.0 |
| Aprimira | 33.4 ± 17.9 | 11.3 ± 7.6 | 1.2 ± 0.9 | 8.1 ± 4.3 | 1.1 ± 0.9 | 4.2 ± 2.0 | 21.1 ± 6.3 |

[a]Milligrams per 100 g fresh weight (FW); mean of three replicates ± standard deviation; minor components are given in the Supporting Information, Table 2S.

**Table 6. Hydroxycinnamic Acid Concentrations in the Fruit Skins of Plum Varieties and Clones[a]**

| | neochlorogenic acid | chlorogenic acid | *p*-coumaric acid deriv | cryptochlorogenic acid |
|---|---|---|---|---|
| Cac. Fruchtbare | 377.5 ± 176.2 | 42.8 ± 8.6 | 1.3 ± 0.3 | nd |
| G. G. Reneklode | 739.1 ± 51.3 | 59.5 ± 15.2 | 3.6 ± 0.9 | nd |
| Hoh 6087 | 287.8 ± 111.5 | 66.0 ± 24.5 | 2.2 ± 0.7 | 1.9 ± 1.0 |
| Jojo | 136.9 ± 48.0 | 17.4 ± 7.3 | 3.8 ± 1.4 | nd |
| Katinka | 310.8 ± 32.7 | 68.6 ± 5.0 | 1.6 ± 0.3 | nd |
| Krichele D. | 478.1 ± 156.7 | 22.3 ± 7.7 | 5.9 ± 2.0 | nd |
| Miragrande | 772.7 ± 218.0 | 7.1 ± 30.5 | 11.1 ± 2.6 | 0.3 ± 0.3 |
| Ortenauer | 412.7 ± 21.7 | 74.4 ± 11.4 | 2.1 ± 0.2 | nd |
| Pogauner | 73.7 ± 8.2 | 11.4 ± 9.2 | 2.2 ± 2.3 | nd |
| President | 242.7 ± 68.4 | 28.1 ± 11.3 | 3.4 ± 1.2 | nd |
| Topper | 347.2 ± 63.7 | 39.2 ± 15.5 | 1.3 ± 0.3 | 3.6 ± 1.0 |
| Toptaste | 985.6 ± 146.2 | 151.6 ± 15.7 | 13.9 ± 0.5 | 3.2 ± 0.6 |
| Sp. Myrobalane | nd | 0.3 ± 0.2 | nd | nd |
| Tatjana | 55.1 ± 7.9 | 12.6 ± 2.1 | 0.9 ± 0.2 | nd |
| Haferschlehe | 624.0 ± 77.7 | 23.3 ± 2.4 | 11.6 ± 2.6 | nd |
| Songold | 445.2 ± 25.9 | 11.8 ± 3.0 | 6.4 ± 0.7 | nd |
| Wei 252 | 24.0 ± 14.7 | 7.2 ± 4.6 | 2.3 ± 1.3 | nd |
| Wei 261 | 73.6 ± 43.6 | 6.3 ± 3.3 | 1.8 ± 1.0 | nd |
| Wei 256 | 8.7 ± 2.6 | 2.8 ± 0.9 | 0.9 ± 0.3 | nd |
| Wei 266 | 8.4 ± 6.6 | 3.0 ± 2.4 | nd | nd |
| Wei 267 | 30.2 ± 25.0 | 6.5 ± 5.5 | 0.9 ± 0.5 | nd |
| Wei 238 | 434.5 ± 49.7 | 46.6 ± 2.6 | 14.6 ± 1.7 | nd |
| Wei 243 | 72.3 ± 21.0 | 15.3 ± 3.0 | 2.7 ± 0.5 | nd |
| Wei 244 | 169.0 ± 61.1 | 13.6 ± 4.1 | 3.9 ± 1.7 | nd |
| Wei 247 | 248.6 ± 111.9 | 42.2 ± 16.1 | 7.5 ± 2.7 | nd |
| Wei 1660 | 35.5 ± 3.6 | 16.3 ± 3.7 | 0.5 ± 0.1 | nd |
| Pr. Frutic. | 479.6 ± 62.5 | 22.5 ± 5.0 | 0.2 ± 0.1 | nd |
| Aprimira | 404.3 ± 92.3 | 64.7 ± 10.6 | 4.4 ± 0.6 | nd |

[a]Milligrams per 100 g fresh weight (FW); mean of three replicates ± standard deviation; minor components are given in the Supporting Information, Table 3S.

**Table 7. Concentrations of Catechins in the Fruit Skin of Plum Varieties and Clones[a]**

| | catechin | epicatechin |
|---|---|---|
| Cac. Fruchtbare | 1.1 ± 0.7 | 1.1 ± 0.4 |
| G. G. Reneklode | 29.0 ± 10.1 | 4.1 ± 1.4 |
| Hoh 6087 | 0.9 ± 0.3 | 0.7 ± 0.6 |
| Jojo | 0.2 ± 0.1 | 0.3 ± 0.1 |
| Katinka | 0.6 ± 0.4 | 0.6 ± 0.1 |
| Krichele D. | 5.2 ± 4.8 | 2.4 ± 1.7 |
| Miragrande | 8.9 ± 4.7 | 2.9 ± 1.2 |
| Ortenauer | 1.9 ± 0.3 | 0.7 ± 0.2 |
| Pogauner | 3.8 ± 3.6 | 0.5 ± 0.3 |
| President | 1.1 ± 0.5 | 1.8 ± 1.1 |
| Topper | 1.3 ± 0.2 | 0.7 ± 0.2 |
| Toptaste | 8.2 ± 1.8 | 2.1 ± 0.3 |
| Sp. Myrobalane | nd | nd |
| Tatjana | 10.3 ± 0.7 | 0.4 ± 0.1 |
| Haferschlehe | 1.4 ± 0.9 | 1.7 ± 1.0 |
| Songold | 224.7 ± 36.0 | 4.1 ± 1.1 |
| Wei 252 | nd | nd |
| Wei 261 | 1.4 ± 1.7 | 0.8 ± 0.7 |
| Wei 256 | nd | nd |
| Wei 266 | nd | nd |
| Wei 267 | nd | nd |
| Wei 238 | 0.7 ± 0.3 | 0.7 ± 0.3 |
| Wei 243 | 0.3 ± 0.2 | 0.1 ± 0.1 |
| Wei 244 | nd | nd |
| Wei 247 | nd | nd |
| Wei 1660 | 0.2 ± 0.1 | 0.6 ± 0.1 |
| Pr. Frutic. | 2.3 ± 1.9 | 3.7 ± 1.6 |
| Aprimira | 1.0 ± 1.3 | 0.1 ± 0.1 |

[a]Milligrams per 100 g fresh weight (FW); mean of three replicates ± standard deviation.

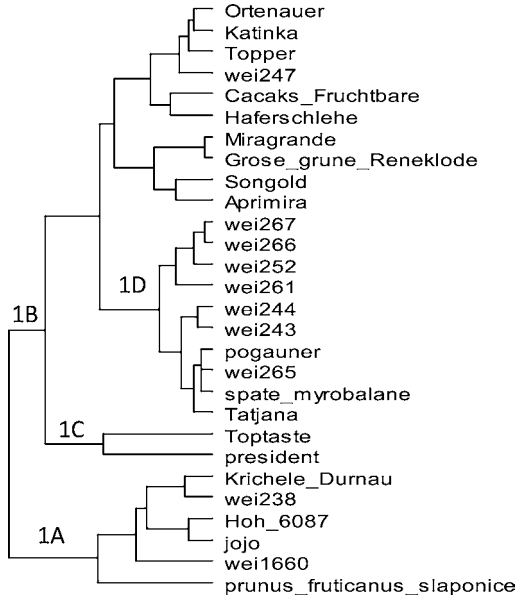


**Figure 3.** Hierarchical dendrogram constructed from phenolics profile as the analytical data (Table 3), illustrating the distances between 28 plum varieties (3 plants per variety).

peonidin 3-rutinoside, as revealed in PCA results (Figure 3; Supporting Information, Figure 1S). Cluster 1B included all other plum samples. 'Toptaste' and 'President', enriched in cyanidin conjugates and neochlorogenic acid, were separated in one small group recognized as 1C. With the exception of 'Tatjana', 'Späte Myrobalane', and 'Pogauner', cluster 1D represents the complete 'Wei' series, which are all progeny from *P. domestica* cultivar 'Jojo'. The close relationship of these varieties could be demonstrated by their low phenolic contents (Figure 1).

With respect to PCA and hierarchical cluster analysis results, the use of phenolic fingerprints revealed the close relationship among different species, but could not identify the hybrids of *P. domestica* × *P. cerasifera* from *P. domestica* × *P. spinosa*. In other words, it was not possible to use phenolic fingerprints to identify the parents in the case of the particular interspecific hybridization. With the effective segregation of samples for 'Prunus Fruticanus Slaponice', 'Krichele Durnau', 'Wei 1660', 'Wei 238', 'Hoh 6087', and 'Jojo' from the plum data set, we tested if the multivariate statistical analysis can still distinguish most other samples still clustered close together. Our goal was also to help identify variation in minor phenolics, aside from neochlorogenic acid, cyanidin, and peonidin conjugates, showing the highest variance across samples. PCA was performed excluding these five metabolites (five variables, namely, cyanidin rutinoside, peonidin rutinoside, cyanidin glucoside, peonidin glucoside, and neochlorogenic acid) along with the most distant samples ('Prunus Fruticanus Slaponice', 'Songold', 'Krichele Durnau', 'Wei 1660', 'Wei 238', 'Hoh 6087', and 'Jojo') from the data set.

The main PC to differentiate plum samples, that is, PC1, accounts for 51% of variance. The PC1/PC2 scores plot (Supporting Information, Figure 2S, A) shows now those three major distinct clusters are formed corresponding to the 19 different samples included. On the left side of the plot, samples 'Pogauner', 'Späte Myrobalane', and 'Wei' samples 'Wei 244', 'Wei 261', 'Wei 243', 'Wei 252', 'Wei 266', and 'Wei 256' are positioned (negative PC1 values), whereas on the far right side, 'Cacaks Fruchtbare', 'Wei 247', 'Tatjana', and 'Hoh 6087' are located (positive PC1 values). The other samples, 'Große Grüne Reneklode', 'Katinka', 'Aprimira', 'Ortenauer', and 'Miragrande', are spread between. This group can still be separated along PC2. Examination of the loadings plot suggested that the variables referred to rutin, quercetin, and *n*-chlorogenic acid contributed the most to the discrimination of samples (Supporting Information, Figure 2S, B).

It could be shown in this study that quantitative phenolic profiles of the fruit skin can be used to differentiate plum varieties. However, cluster analysis did not simply separate varieties of the European plum *P. domestica* from the species *P. cerasifera*, *P. spinosa*, and *P. salicina*, which may confirm their close relationship and the hybrid nature of the European plum. To use phenolic fingerprints for a study of the genetic relationship among varieties and *Prunus* species, a greater number of samples from the respective species as well as populations of known origin and defined crosses have to be analyzed.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Concentrations of minor and tentatively identified phenolic compounds as well as PCA scores plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Funding

M.A.F. acknowledges the Alexander von Humboldt foundation for financial support. M.A.F., D.W., and G.D.A.B. are grateful to the European Community for an Erasmus Mundus fellowship.

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We thank Anja Forstner for technical assistance.

## ■ DEDICATION

This paper is dedicated to our young colleague Gisselle Doris Argueta Baires, who abruptly died during preparation of the manuscript.

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