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Characterization of *Prunus avium* L. Varieties with Phenolic Compounds

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Abstract

Phenolic compounds of 23 sweet cherry varieties, including some local selections of the same variety, were analyzed to check their identity or distinctness. The compounds were extracted from the inner bark of one year old shoots and separated by HPLC (High Performance Liquid Chromatography). The high discriminatory potential of phenolic compounds for variety identification could be shown. Even different origins of the same variety could be identified. The results of the polyphenol analysis showed in many cases good accordance with morphological classification, whereas for a few varieties a revised nomenclature seems to be necessary.

Introduction

A large field of discussion for sweet cherry growers is the correct and definite identification of varieties, since they are often morphologically very similar. Even experts have problems positively identifying varieties, although there are a lot of morphological markers for their characterization (1). Additionally, some varieties are genetically not uniform, but are a mixture of different types (7). This has led to many local varieties with different names. Already the "classical" pomologists, as Truchsess (21) and Jahn (11) found inconsistency and synonyms for the same variety. "Modern" pomologists are able to detect new characters by biochemical methods. The classical method still is the electrophoretic separation of proteins and isoenzymes extracted from various tissues, which results in, variety specific protein bands. This technique has been used for the identification of varieties for many plant species, including sweet cherry (14), apple (12, 23), raspberry (6), apricot (5) and grape (17). The main problem of this technique is the lack of a clear and definite nomenclature pattern for protein bands. The only criteria is the position of the band on the gel, whereas function and structure are unknown. So a simple comparison of protein bands gives only poor information how distinct or conform two genotypes are. Additionally, the evaluation of data is only semi-quantitative.

Therefore, for the present investigation, the analysis of phenolic compounds by HPLC was chosen. Using UV-spectra and retention time, it can be decided very precisely, whether two varieties are qualitatively different or whether a peak with the same retention time is actually the same compound.

The quantity and quality of phenolic compounds has been used as criteria for variety identification since 1980. Phenolic compounds occur in almost all higher plants, they show a high structural diversity (16), even within different tissues (bark, leaf, flower, pistil, pollen) and are therefore best suited as taxonomical markers (4, 8, 13, 22). Henning and Herrmann (10) were the first to find varietal specific phenol composition in leaves and fruits of sweet cherries. Treutter and Feucht (18) showed that variety identification can also be carried out by phenolic compounds of the inner bark. Phloem, especially winter phloem bears several advantages compared to leaves or

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fruits: low physiological activity and little environmental influence. Phloem samples can be taken during the whole year and chemotaxonomical investigations are possible even in the juvenile period of a tree. Several varieties which are typical for the South German sweet cherry growing area of Franconia were investigated for the present study to verify the identity of variety names.

Materials and Methods

All plants were grown in the same orchard in Franconia. Varieties were grafted on F12/1 in 1978. In March 1991, 3 one year old shoots from the same tree were cut (3 samples for each variety), stored immediately at -30° C and afterwards lyophilized. The outer bark (epidermal layer, phellem, phellogen, phelloderm, bast fibers) was removed and the phloem scraped off. 100 mg of this powder was extracted with 20 ml cold 80% aqueous acetone containing 0.1% 6-methoxy-flavone (internal standard) in a sonicator for 30 min. After filtration the solvent was evaporated and the residue dissolved in 2 ml methanol. 5 μl of this solution were injected into the HPLC. Chromatographic parameters and peak identification have been described elsewhere (8, 9).

The equipment for the analytical HPLC consisted of a low pressure gradient system (gradient fromer model 250B and HPLC pump model 300CS, Gynkotek) a photodiode array detector (Hewlett Packard 1040), a work station (Hewlett Packard 9000), a sample injector (Abimed, model 231) and a reversed-phase (RP) C_{18} 3 μm column (Shandon ODS, 4.6 x 250 mm ID). Solvent A was 1% aqueous acetic acid, solvent B methanol/butanol (5:1).

23 varieties/selections, most of them are local varieties of Franconia, were analyzed (see Table 1). The genealogy of these varieties is poorly known. Most of them were selected from seedlings over periods of time because of some desired characters.

Gradient range:		
0- 10 min	8% B isocratic	
10- 20 min	8-10% B	
20- 30 min	10-14% B	
30- 40 min	14-17% B	
40- 50 min	17-20% B	
50- 60 min	20-27% B	
60- 70 min	27-33% B	
70- 80 min	33-49% B	
80- 90 min	49-62% B	
90-100 min	62-80% B	
100-110 min	80% B isocratic	

Flow rate: 0.5 ml/min
Detection: 280 nm
Injection: 5 μl

Statistical analysis:

Discriminant analysis was calculated to discriminate among varieties according to their phenolic pattern (18 compounds). Each variety was treated as a single group (23 groups). Varieties were compared two by two by Scheffé's-Test, using the mean value of each discriminant function. Varieties were regarded to be different, when at least one of the 22 discriminant values, which are calculated for each group (variety), had a different range at the 5% level.

Hierarchical cluster analysis was used to find relationships among varieties according to their phenolic pattern (18 compounds). Each replicate was treated as a single case. Agglomeration was carried out using Ward's Method, distances between each case calculated as Squared Euclidean Distances.

Furthermore confidence intervals were calculated to compare mean values of each peak between varieties.

Results and Discussion

I. Phenolic Compounds

Figure 1 shows a HPLC chromatogram of phloem phenols of variety 'Hedelfinger.' In this tissue about 70 different phenolic structures are present. 18 of them (peaks No. 3, 5, 11, 13, 18, 19, 21, 23, 25, 27, 28, 33, 35, 36, 37, 41, 42 and 44) could be used for the identification of varieties, since all others are present only in trace amounts or are not well separated from each

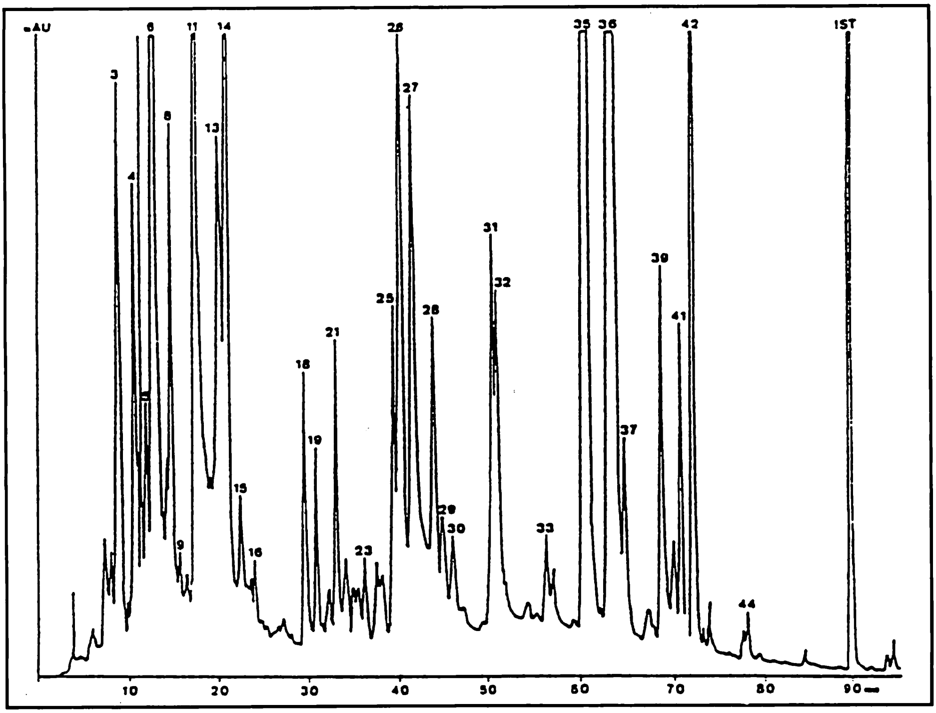

Figure 1. HPLC-separation of phloem phenols of variety 'Hedelfinger,' detection at 280 nm.

Legend (common name and structural name in parentheses):
Peak 6 = Catechin (3,3',4',5,7-Flavanpentol)
Peak 11 = p-Coumaroyl-glucose
Peak 18 = Dihydrokaempferol-7-glucoside (3,4',5,7-Tetrahydroxyflavanone-7-glucoside)
Peak 21 = Eriodictyol-7-glucoside (3',4',5,7-Tetrahydroxyflavanone-7-glucoside)
Peak 26 = Genistein (4',5,7-Trihydroxyisoflavone-7-glucoside)
Peak 29 = Prunin (4',5,7-Trihydroxyflavanone-7-glucoside)
Peak 31 = Sakuranin (4',5-Dihydroxy-7-methoxyflavanone-5-glucoside)
Peak 32 = Genistein-5-glucoside (4',5,7-Trihydroxyisoflavone-5-glucoside)
Peak 33 = Apigenin-7-glucoside (4',5,7-Trihydroxyflavone-7-glucoside)
Peak 35 = Cherry-factor (structure unknown)
Peak 36 = Dihydrowogonin-7-glucoside (5,7-Dihydroxy-8-methoxyflavanone-7-glucoside)
Peak 37 = Isosakuranin (5,7-Dihydroxy-4'-methoxyflavanone-7-glucoside)
Peak 39 = Genistein (4',5,7-Trihydroxyisoflavone)
Peak 40 = Naringenin (4',5,7-Trihydroxyflavanone)
Peak 42 = Chrysin-7-glucoside (5,7-Dihydroxyflavone-7-glucoside)

other. The dominating compound of the phloem is peak No. 36, dihydrowogonin-7-glucoside, which represents about 25% of the total phenol content.

All sweet cherry varieties showed qualitatively an identical phenolic pattern. The differences between them were found to be quantitative. By statistical comparison most of the 23 varieties could be distinguished (Table 2). No significant difference could be observed between these pairs of varieties: 'Abels Späte I'-'Froschmaul,' 'Abels Späte I'-'Hedelfinger,' 'Froschmaul'-'Hedelfinger,' 'Brocken'-'Souvenir de Charmes,' 'Burgheimer'-'Haumüllers Speck,' 'Haumüller I'-'Haumüller II,' 'Haumüller I'-Variety No. A, 'Haumüller II'-Variety No. A and 'Schmahlfelds Schwarze I'-'Schmahlfelds Schwarze II.'

The relationship among all varieties, basing on the phloem phenol composition, can be seen in a cluster analysis (Figure 2). Those varieties which have a very similar phenol pattern are unified at a low level into one cluster. The more the cluster distance increases,

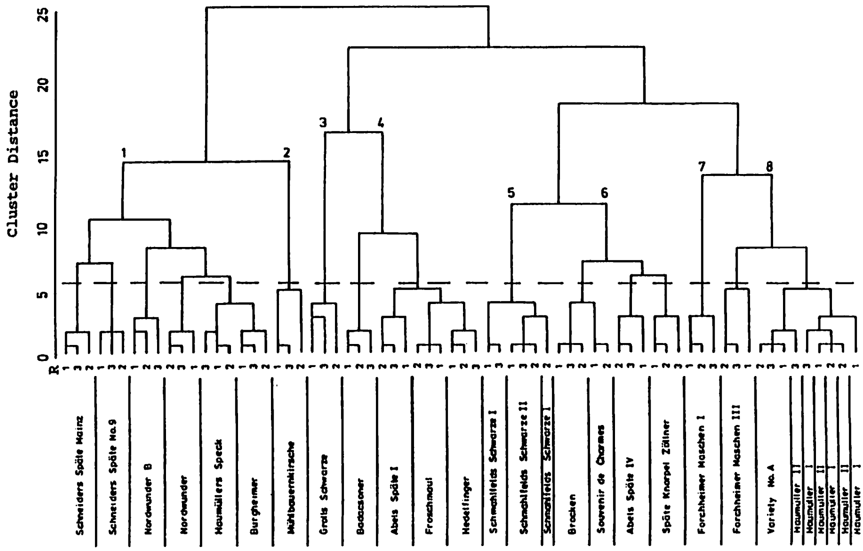

Figure 2. Dendrogram of a cluster analysis on the basis of 18 phenolic compounds. Variation below cluster distance 5 (dotted line) is due to environmental influences, above this level significant differences among varieties are observed (R = Number of Replicate, numbers in the dendrogram indicate cluster number).

Table 1. list of varieties and some of their important morphological characters (different numbers or letters refer to different origins or selections of the same variety).

Variety	Age of Maturity (given in weeks, relative to 'Burlat')	Flowering Time I = early II = middle III = late	Remarks
Souvenir de Charmes Brocken	2-3 2-3	I I	Local variety of Franconia; selection out of Souvenir de Charmes
Forchheimer Maschen I Forchheimer Maschen III	3	I	Presumably selected out of Knauffs; no clear differences between the two types during cultivation
Schmahlfelds Schwarze I Schmahlfelds Schwarze II	4 4	III III	Selections out of Schmahlfelds; no clear differences between the two types during cultivation
Grolls Schwarze	3-4	II	These varieties are morphologically clearly separated from all others
Badasconer	4-5	I	
Späte Knorpel Zöllner	5-6	III	
Haumüllr 1 Haumüller 2	4-5	II	Local variety of Franconia; samples were taken from two different trees (1 and 2)
Variety No. A	4-5	II	Identity not clear, presumably confusion of scions with Haumüller; similar in all morphological characters to Haumüller
Schneiders Späte (Selection Mainz) Schneiders Späte (Selection No. 9) Nordwunder Nordwunder B	5-6 5-6 5-6 5-6	II II II II	All members of the Schneiders-group are morphologically and physiologically similar Selection out of Nordwunder
Burgheimer Haumüllers Speck Mühlbauernkirsche	5-6 5-6 5-6	II II II	Local varieties of Frnaconia; origin not clear, presumably 'Schneider'-types; all varieties were grafted on the same tree
Hedelfinger (type Diwmitz) Abels Späte I Abels Späte IV Froschmaul	5 6-7 5-6 6	III III III III	 Local variety ;of Franconia; Hedelfinger-type; Hedelfinger-type; morphologically clearly different from type I (concerning way of growth and fruit characters) Local variety of Franconia; Hedelfinger-type

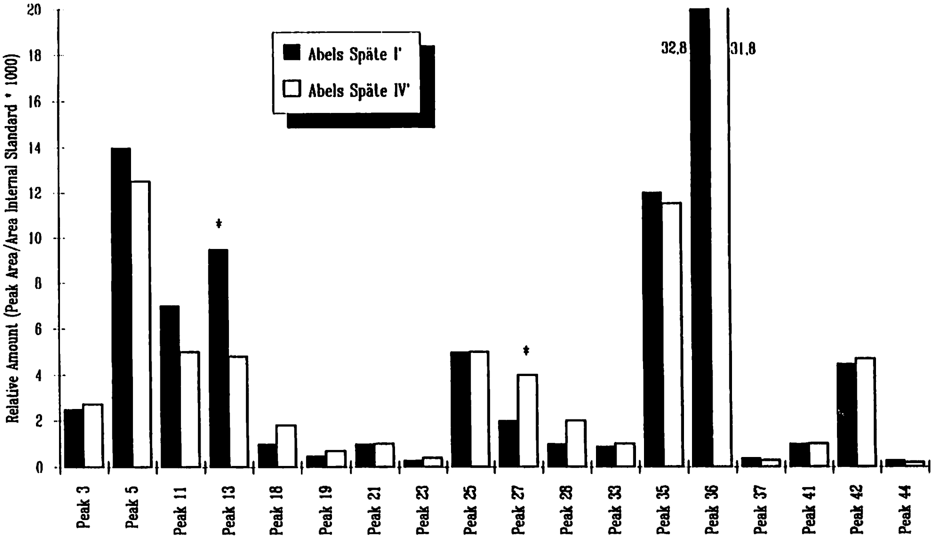

Figure 3. Mean values of peak area of the origins from 'Abels Späte.' Different concentrations (confidence interval, α = 5%) were found for compounds No. 13 and 27.

the more differences between these varieties exist. Varieties segregated into 8 general clusters:

Cluster 1: 'Schneiders Späte Mainz,' 'Schneiders Späte No. 9,' 'Nordwunder B,' 'Nordwunder,' 'Haumüllers Speck,' 'Burgheimer'
Cluster 2: 'Mühlbauernkirsche'
Cluster 3: 'Grolls Schwarze'
Cluster 4: 'Badasconer,' 'Abels Späte I,' 'Froschmaul,' 'Hedelfinger'
Cluster 5: 'Schmahlfelds Schwarze I,' 'Schmahlfelds Schwarze II'
Cluster 6: 'Brocken,' 'Souvenir de Charmes,' 'Abels Späte IV'; 'Späte Knorpel Zöllner'
Cluster 7: 'Forchheimer Maschen I'
Cluster 8: 'Forchheimer Maschen III,' Variety No. A, 'Haumüller I,' 'Haumüller II'

This classification is in many cases in good accordance with morphological and genetical data. 'Schneiders' (Cluster 1)—and 'Hedelfinger' (Cluster 4)—types can be clearly separated. 'Burgheimer' and 'Haumüllers Speck' are related to the 'Schneiders'-, 'Badasconer' to the 'Hedelfinger'-group. 'Mühlbauernkirsche' and 'Grolls Schwarze' are placed between, but are clearly distinct.

As indicated by discriminant values, the origins of 'Schmahlfelds Schwarze' and both samples of 'Haumüllers' are close together. Against that, both types of 'Abels Späte' are clearly separated. The same is true for the selections of 'Forchheimer Maschen.' They are clearly different and unified at a high level into one cluster.

Cluster No.6 comprises a very heterogenous group of varieties. The origin of 'Brocken' from 'Souvenir de Charmes' can be seen, since both varieties are clustered close together. The unknown variety No. A is placed together with both trees of 'Haumüller' and 'Forchheimer Maschen III' into cluster No. 8, but being clearly related to 'Haumüller.' Since all samples which have been analyzed for 'Haumüller' are unified in one cluster at a cluster distance of 5, we would suggest, that above this level significant differences among varieties exist, whereas variation below this level is due to environmental influences. Following this rule, and regarding the results of the

Table 2. Two by two comparison of discriminant values by Scheffé-Test (* significant, $\alpha = 5\%$).

	Abels Späte I	Abels Späte IV	Badasconer	Brocken	Burgheimer	Forchheimer Maschen I	Forchheimer Maschen III	Froschmaul	Grolls Schwarze	Haumüller I	Haumüller II	Haumüllers Speck	Hedelfinger	Mühlbauernkirsche	Nordwunder	Nordwunder B	Schmahlfelds Schwarze I	Schmahlfelds Schwarze II	Schneiders Späte Mainz	Schneiders Späte No. 9	Souvenir de Charmes	Späte Knorpel Zöllner	Variety No. A
Abels Späte I																							
Abels Späte IV	*																						
Badasconer	*	*																					
Brocken	*	*	*																				
Burgheimer	*	*	*	*																			
Forchheimer Maschen I	*	*	*	*	*																		
Forchheimer Maschen III	*	*	*	*	*	*																	
Froschmaul		*	*	*	*	*	*																
Grolls Schwarze	*	*	*	*	*	*	*	*															
Haumüller I	*	*	*	*	*	*	*	*	*														
Haumüller II	*	*	*	*	*	*	*	*	*														
Haumüllers Speck	*	*	*	*		*	*	*	*	*	*												
Hedelfinger		*	*	*	*	*	*		*	*	*	*											
Mühlbauernkirsche	*	*	*	*	*	*	*	*	*	*	*	*	*										
Nordwunder	*	*	*	*	*	*	*	*	*	*	*	*	*	*									
Nordwunder B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*								
Schmahlfelds Schwarze I	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*							
Schmahlfelds Schwarze II	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*							
Schneiders Späte Mainz	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*					
Schneiders Späte No. 9	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*				
Souvenir de Charmes	*	*	*		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*			
Späte Knorpel Zöllner	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		
Variety No. A	*	*	*	*	*	*	*	*	*			*	*	*	*	*	*	*	*	*	*	*	

Scheffé-Test close relationships are found for Variety No. A and 'Haumüller,' 'Souvenir de Charmes' and 'Brocken,' 'Schmahlfelds Schwarze I,' and 'Schmahlfelds Schwarze II,' 'Abels Späte I,' 'Froschmaul' and 'Hedelfinger' and finally 'Haumüllers Speck' and 'Burgheimer'.

II. Comparison of biochemical data versus morphological characters

'Mühlbauernkirsche,' 'Grolls Schwarze' and 'Badasconer' are clearly distinct from each other and from all other varieties. Phenol analysis confirms these morphological observations, with 'Badasconer' being distantly related to the 'Hedelfinger'-group.

'Schneiders Späte' is frequently used as a synonym for 'Nordwunder' (20). For the growers both varieties are almost identical. The phenol pattern of these varieties was similar, but one or two compounds had different concentrations. Therefore 'Nordwunder' is related to the 'Schneiders'-group but not identical with 'Schneiders Späte'. Both selections of 'Schneiders

Späte' and 'Nordwunder' showed a different phloem phenol pattern. Together with 'Haumüllers Speck' and 'Burgheimer,' which are indistinguishable from each other, they form a complete "Schneiders-cluster" (cluster No. 1), confirming growers observations, that all varieties of this group have a lot of similar characters.

Sometimes there is a confusion, whether 'Haumüller' is a synonym for 'Schneiders Späte'. But the 'Haumüller'-genotype investigated here was not related at all to the 'Schneiders'-group.

With the exception of 'Abels Späte IV,' cluster No. 4 represents a complete 'Hedelfinger'-cluster. 'Froschmaul' and 'Abels Späte I' are 'Hedelfinger'-"sports" (24). They ripen one week after 'Hedelfinger'. The close relationship of all these varieties could be demonstrated by their phenolic constituents. The origin of "sports" by phenolic compounds has already been shown (3), although "sports" are morphlogically or physiologically (e.g. age of maturity) clearly different from their corresponding mother plant. Nevertheless, their phenolic composition can be identical, since "sports" are not the result of a new combination of the genetic material. Often they differ from their mother plant only in one character. Therefore, single mutations in the somatic tissue can be without any influence on the phenol pattern. Phloem phenol metabolism seems to be very stable and relatively unaffected by spontaneous mutations.

'Abels Späte I' and 'Abels Späte IV' can be clearly distinguished by their phenol pattern (Figure 3). This finding agrees with practical experiences, since both types are easily distinguishable by the growers with respect to their fruit characters.

Both types of 'Forchheimer Maschen' show a different phenol pattern. This is totally contradictory to practical experiences, for there are no pronounced differences between them. Presumably they differ in some physiological characters, which are not visible for growers. Therefore, the actual nomenclature should be maintained.

Against that, there seems to be no reason to treat the selections of 'Schmahlfelds Schwarze' (cluster No. 5) as individual genotypes. There are either no morphological nor any "phenolic" differences. The unknown Variety No. A is identical to 'Haumüller' by its phenolic constituents. This confirms growers observations, that there must have been a mix up of scions. Biochemical analysis clearly revealed its identity.

Conclusion

The high discriminatory potential of phenolic compounds for variety identification could be shown. Nevertheless, when working with these compounds, it is extremely important to control environmental influences (2, 15). Especially the age of a tree and the location modifies the phenolic composition of *Prunus avium* phloem, whereas phenol metabolism of one and two year old shoots was relatively unaffected (19). Therefore, only scions of one year old shoots from the same rootstock were taken. Samples were harvested in winter, when physiological activity is at a minimum (18). To control environmental influences, two samples of the same variety ('Haumüllers') were taken from two different trees. Genetical differences among varieties clearly superimposed environmental effects.

In cases where the classification of varieties is uncertain, the data of phenol analysis are mostly in good agreement with morphological observations of growers, but some modifications of the actual nomenclature are indicated: both selections of 'Schmahlfelds Schwarze' should be unified as one single genotype. The same is true for 'Haumüllers Speck' and 'Burgheimer' on the one hand, and 'Brocken' and 'Souvenir de Charmes' on the other hand. The unknown variety is identical to 'Haumüaller.'

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