DIPLOMARBEIT

Resveratrol in shoots, stems and leaves from Vitis vinifera

ausgeführt am

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“The significant problems we face cannot be solved at the same level of thinking we were at when we created them” (Albert Einstein)

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Abbreviation List

FP7:	Frame Program 7
BF:	Blaufränkisch
GV:	Grüner Veltliner
RR:	Rheinriesling
ZW:	Zweigelt
EPEA:	Environmental Protection and Encouragement Agency
CT:	catechin
CIS-PIC:	cis-piceid
CIS-RES:	cis-resveratrol
TRANS-:	trans-piceid and trans-resveratrol
RESVE:	resveratrol dimer
E-VINIF:	ε-viniferin
VINIFER:	viniferin 1, 2 or 3
p.a.:	pro analysi
st A:	stems April
st J:	stems July
st S:	stems September
le J:	leaves July
le S:	leaves September
sh J:	shoots July
sh S:	shoots September
Sept:	September
conc.:	concentration

Abstract

Trans-resveratrol is a highly researched component that has many health qualities. It is one of the most potent natural antioxidants produced in plants in stress conditions. It is well known in wine, but only little knowledge is available about its concentration inside the rest of the plant. A comparison of shoots, leaves and stems in the four main Austrian vine cultivars, Blaufränkisch, Grüner Veltliner, Rheinliesling and Zweigelt was taken out. The amounts show no specific trends to certain cultivars nor to the colour of the grapes. Great differences can be observed in the different parts of the plants showing the lowest amounts in leaves at around 0,012 mg/g dw and the highest in stems, up to 1,7 mg/g dw.

1 Introduction

Circular flow stream management is a whole new branch in our economic system. Products may never have an ending life, it is an immortal view of mass. Waste as we knew it, may no longer exist and neither is the end of growth, the only solution.

The worldwide economy is broadly based on non stop pollution to work. The concept of our system is take - make - waste which obviously leads sooner or later to a devastated planet. If everything which is produced, for use of a period from hours to months becomes waste, which needs again centuries or more to decay, the earth will collapse under this endless mass of junk.

The idea of generating value from products that seem to have reached their end of the line is not new at all but often have been forgotten. This study in sampling leftovers was about gaining an added value per land unit of viticulturally exploited land, through the implementation of forward-looking utilization methods during the entire life-cycle of the cultivated vitis-biomass, in terms of an advanced material flow management approach. The focus should be on the utilization of higher-weight molecules and premium secondary plant substances by means of gentle state-of-the-art extraction / separation procedures in the sense of biocascading, a screening of accruing recycled fibre stock within viticulture. Besides the multiple use of above-ground plant material from vine, the industrial utilization of intercrops (mixed culture) and additional renewable energy production by intercropping and secondary biomass conversion, a production of high-value fertilizer to maintain soil fertility must also be employed, following the cradle to cradle™ strategy [1].

1.1 Cradle to Cradle

Michael Braungart from the EPEA Institute in Hamburg is the initiator of this new design principle. Celebrating Diversity is the maxim, with integrating high demands on the material flow, being the standard. Waste equals food replaces the old paradigm, from cradle to grave. The certification criteria are long and are about identifying all material components, defining them as biological or technical nutrients and substituting all harmful components whatsoever. It is not about reducing or minimizing, as optimizing the wrong system components is efficiently wrong [2].

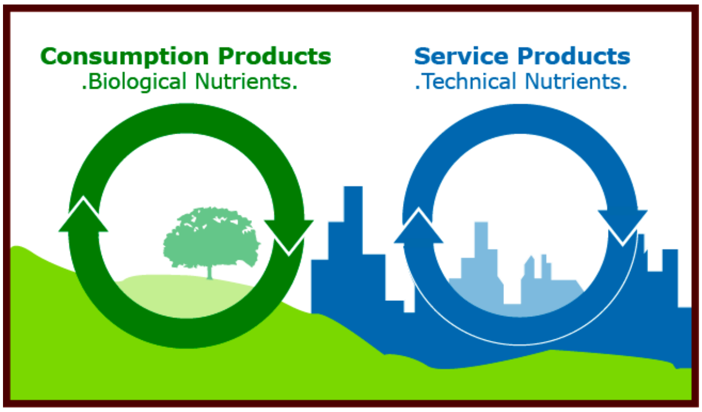


Figure 1: Biological and Technical Metabolism

1.2 Viticulture

Vineyards cover more than 8.000.000 ha worldwide, characteristically defining the landscape. This cultural heritage maybe has one of the greatest impacts in all classes of the population; also on researchers with ideas to maximize income in one way or the other. There is pure scientific research on ingredients or even molecular biological levels and applied research on the crop itself and all kinds of outside influences.

For the concern of this study, influences on the resveratrol concentration in vines, were from deep interest. The cultivation methods, considering the rootstock of the vine where the locally adapted variety is grafted, have not been reviewed so far on the distribution of polyphenolic compounds. If the soil properties change, the polyphenolic compounds are affected, as is described in [3]. With the addition of organic matter as fertilizer, the anthocyans as well as the total polyphenolic index may decrease. The topography of the vineyard plays a central role in the production of polyphenols, as resveratrol biosynthesis is induced by ultraviolet irradiation [11]. One factor is the angle of the cultivated land with its exposure possibilities to the sun. With a perpendicular angle to the sun rays, the vine can receive greater strength than in flatter terrain, where the sunlight is diluted. There is also the drainage effect that slopes offer, so the vine does not have too much moisture in the soil. A higher water-holding capacity of the soil leads to a significantly lower colour intensity of the wine, as well as lower total polyphenolic compounds [4]. The opposite takes place with drought conditions.

Considering the tendering measures, thinning out the bushes leads to more concentrated wine with less free polyphenols but more polymerized polyphenols [5].

The harvest time may also have an effect on the total polyphenol and resveratrol content, with later harvests yielding higher concentrations in the wine itself, due to more intense stress factors. On the contrary, the sorting out of affected grapes will lessen these contents. The contents in question do not differ significantly in wines vintagewise or according to the wine-growing sub-region or wine kind [6]. The same authors found out that within a period of five years the general amount of trans-resveratrol in all measured samples has declined and assume that global warming takes an effect on the northern regions of the Czech Republic. Warmer and drier weather conditions lower the development of grey mould and therefore also the amount of secondary plant compounds produced, especially resveratrol in the plant [7, 9, 8].

Warm climate grapes with high sugar contents may favor the glycosylation of resveratrol to the piceid or also called polydatin [12].

Resveratrol is synthesized mostly in response to disease or injury stress [25, 26, 18]. The anti-phytopathogenic properties of trans-resveratrol and trans-ε-viniferin are widely described in the literature, against downy mildew (*Plasmapora viticola*) [13, 14, 15, 16], grey mould (*Bortrytis cinerea*) [14, 19, 20, 17, 18], *Phoma medicaginis [21], Rhizopus stolonifer [22],* and a broad spectrum of microbes and fungi present during postharvest fruit storage [23, 24]. Application of a very low concentration of resveratrol in water to stored fruits does not only keep the infection rate down for almost triple the time of storage, but also no signal of losses or deterioration can be detected and the postharvest quality of the fruits is maintained. Sierra Rayne [27] states that there is a potential to utilize grape cane extracts as anti-phytopathogenic sprays to aid in on-farm sustainability. Such a conceptual approach involves the replacement of synthetic chemical analogs and the associated environmental and economic costs. In order to grow high quality grapes the vineyard needs many tendering measures throughout the year producing an interesting amount of plant leftovers, only the weight of the lignified canes, that are cut away each year amount to 1000 to 3000 kg per hectare [28]. They represent a potentially important global source of trans-resveratrol and trans-ε-viniferin. After extraction, the residues could be still used for other value-added purposes. There have been two reports on stilbenes in vine [29, 30] so far, where 0,1 - 4,7 mg/g dry weight trans-resveratrol contents in samples from six *Vitis vinifera* cultivars were measured, with levels increasing throughout the growing season.

The comparison from conventional and organic grape production show expected behaviour in terms of the amount of phenolic compounds[10], where the amount is usually higher in organic cultivation. In this case no chemicals should be applied to the field and the self-defense mechanisms of the plant are encouraged. The resveratrol content can also be lower after fungal infection, though expression is high, the consumption of the fungus may even be higher [23], which could be due to a fungus enzyme that can degrade resveratrol completely [8, 31].

1.3 Resveratrol

Resveratrol (3,5,4'-trihydroxystilbene) is a phytoalexine present in vines, peanuts and japanese knotweed (*Polygonum cuspidatum*). It is one of the most well-known stilbenes, the biosynthesis of which is induced in many ways, as described above.

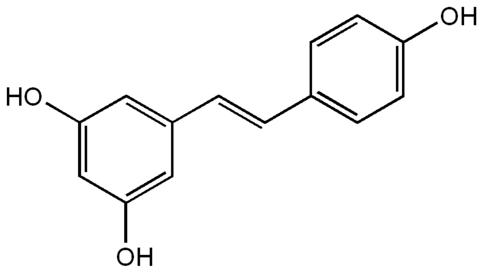


Figure 2: Trans-resveratrol

The 3-β glucoside, trans-piceid or polydatin as a major resveratrol derivate is also common in grapes and could even exceed the amount of resveratrol in fresh grape juice or red wine [32]. The hydrolysis to the bioactive trans-resveratrol occurs with lactase phlorizin hydrolase or cytosilic-β-glucosidase in the small intestine and liver [33].

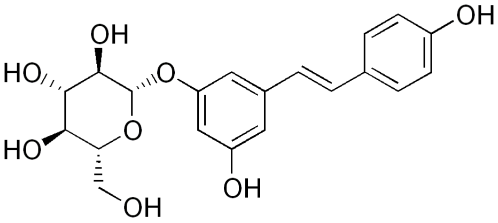


Figure 3: Trans-piceid

As a monomer it exists in two possible isomers, cis- and trans-resveratrol. The cis isomer is built after the exposure of trans-resveratrol to sunlight [34]. The wavelength for the maximum absorption of the two isomers differs from around 304 nm for trans-resveratrol to 286 nm for cis-resveratrol [35]. Pure trans-resveratrol [36] in hydro-alcoholic and pure alcoholic solutions is stable in the dark, whereas exposure to ultraviolet irradiation for 5 days leads to a conversion of 67% from trans- to cis- resveratrol and to the according wavelength shift for the maximum absorption. It is also described that separation of the two isomers is possible with HPLC and a C18 stationary phase due to the proximity of the two phenyl rings in cis-resveratrol and the strengthening of the weak hydrophobic interactions with the stationary phase of the chromatographic column.

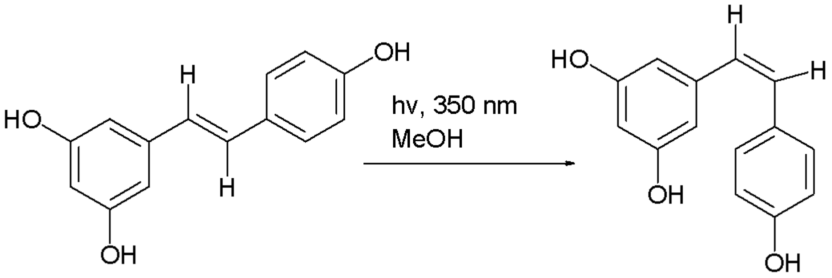


Figure 4: Isomerization of trans- to cis-resveratrol

Oxidative polymerization in the plant produces oligomers of trans-resveratrol, the so called viniferins. Dimers, trimers and tetramers and their glucosides have been identified in the genus Vitis[54, 30]. They are proven to be active ingredients as well and may even have higher potential in positive properties according to human health aspects. The main dimer in vine is said to be ε-viniferin.

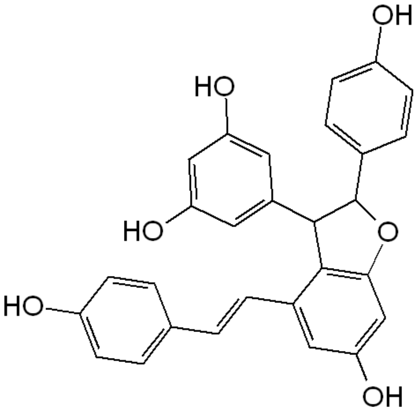


Figure 5: ε-viniferin

The impact of this compound is widespread where antibacterial, fungicide, antitumor and caridioprotective affects have been researched [37]. It was in the centre of interest for many years due to its presumed healthy qualities and the relation of resveratrol to the "French paradox", which portrays the higher intake of saturated fats in France and a relatively low incidence of coronary heart disease. It was correlated to the high consumption of red wine with all the active ingredients and a special attention on resveratrol as the main active component [38].

Trans-resveratrol is the first member of the stilbene series to be synthesized in plants by the enzyme stilbene synthase, via the shikimic pathway, right after the second aromatic ring closure with malonyl-CoA [55].

There have been almost 10000 research approaches on resveratrol directly or indirectly. Especially in China, hundreds of publications have been taken out recently. Prices are high, with a cost of at least 100€ per 100 milligram [39] and new extraction sources would be attractive.

1.3.1 Health aspects

Recent research work shows the ability of trans-resveratrol to inhibit or delay a wide variety of diseases [40], including cardiovascular disease [41] and cancer [42, 18]. In rats, inhibition of platelet aggregation and LDL oxidation and protection of liver from lipid peroxidation by resveratrol were reported [44, 45]. Suppression in the growth of cancerous cells from breast cancer patients by resveratrol was reported to come from apoptosis, programmed cell death [46]. It is said to increase stress resistance and lifespans [40, 43].

Grape seed proanthocyanidin extract contain about 5 mg/g trans-resveratrol which induces the expression of vascular endothelial growth factor (VEGF) in keratinocytes, it could be used to treat dermal wounds and other dermal disorders [47].

Trans-ε-viniferin has been shown to have hepatoprotective [48] and antioxidant [49] properties, to induce apoptosis of leukemia B-cells [50], and inhibit human cytochrome P450 enzymes [51], noradrenaline and 5-hydroxytryptamine uptake, and monoamine oxidase activity [52].

1.3.2 Analytical methods

1.3.2.1 Extraction

The extraction processes are mostly described with ethanol or methanol mixtures. Afterwards separation through centrifugation and the liquid part is concentrated under vacuum at low temperature. A patented description from *Polygonum cuspidatum* uses hexane or diethyl ether as a solvent [57].

Another extraction method described in a patent about hydroxystilbenes[58] uses either watery solvents with a pH at about 11,5 or polar-aprotic organic solvents or even mixtures. After many hours of percolating extraction with the watery solvent an acidation to a low pH at around 3,5 will lead to a precipitation of the hydroxystilbenes, which will be cleaned and dried afterwards. When using organic solvents the cleaning can be provided with chromatographic means or even separation of the solvent.

Pezet et al[60]suggests extraction of crushed grape berries with methanol and after centrifugation adding of water to the supernatant. This solution is pre-purified by solid-phase extraction and eluates collected also from column washing procedure with methanol-water. The combined eluates are evaporated in a water bath under a nitrogen stream. This water solution is extracted with diethyl ether three times, the organic fraction then dried, filtered and again evaporated with nitrogen. The residue is then solubilized in methanol and injected into the HPLC analytical system. Here and in the research work of Hart et al [62] solvent evaporation steps are done under nitrogen and protected from light.

Waterhouse et al [61] describes grape berry skins to be extracted by blending in an alcoholic solution of MeOH-EtOH (90:10). The extract is washed twice with petroleum ether and evaporated to a syrup, which is then dissolved in NaCl saturated water. EtOAc is added and separation of the two phases follows where the aqueous phase is adsorbed on a C-18 cartridge, washed with H_2O and the piceid is eluted with MeOH. The extract is evaporated and the final residue dissolved in H_2O and analysed by HPLC for the piceid. The organic phase is evaporated and redissolved in 1:1 H_2O-Acetonitrile for HPLC analysis of resveratrol. For comparison the piceid was treated with β-glucosidase and resveratrol was evidenced.

Wine is often analysed directly without purification nor extraction.

A recent study from the year 2006 about polyphenol extraction of lignified stems from Vitis vinifera suggests methanol as the solvent and dilution with methanol-water after air-drying for 60 days. Reversed phase HPLC with UV, fluorescence and MS are used for detection [30].

Another study of lignified canes of Vitis vinifera was taken out in 2003, where a mixture of MeOH-H_2O (7:3, v/v) was used as a solvent. Further purification with EtOAc and low pressure C18 column and a preparative HPLC were used [59]. This study mostly concerned viniferins.

1.3.2.2 Analysis

For analysis HPLC is the most common method chosen; also the column for separation seems to be comparatively the same, a RP C-18 with 5 µm particle size, though mobile phases and detection afterwards may differ. Pezet and his colleagues made some effort on this subject, in one of their studies in 1994 [60] the analysis is described with a gradient of the mobile phases with methanol and methanol-50 mM formic acid and ongoing UV detection and describing fluorimetric detection as being more sensitive and specific. Under long wavelength UV light (366 nm) stilbenes show a bright blue fluorescence.

In the vast amount of scientific papers available for resveratrol analysis, UV detection is mostly used, at least for comparison. There are also descriptions for preparative HPLC, for example with methanolic extraction and diode array UV detection [61].

2 Materials

2.1 Chemicals

Methanol: Roth

Water: Fluka

Acetic acid: Roth

Ortho-phosphoric acid: Roth

Formic acid: Merck

Acetonitrile: Roth

Vanillin: Merck

Catechin: Roth

Solvents used for regenerating HPLC column: methanol, water: as described above; sulfuric acid and chloroform: Roth; tetrahydrofuran: Pranochem

The solvents used for HPLC analysis were filtered through a 0,2 µm porous filter: Roth

Trans-resveratrol standard and polydatin were from the Austrian Axxora platform [53], called Eubio.

2.2 Instruments

Grinding mill: Severin KB 30 S, 26KN

Shaking bath: Grant OLS 200

Centrifuge: Heraeus Instruments Labofuge 400R

0,2 µm PTFE porous one way filter units

100 sample flasks, 2 ml, brown glass, teflon sealing: Roth

Spectrophotometer: Beckmann Coulter DU 800

HPLC column: Lichrosorb Reverse Phase C18, 250 mm long, 4 mm wide, the same pre-column, SRD (Säulen Refill Dienst Vienna)

The HPLC was from Perkin Elmer Series 200. It consisted of an autosampler with 10x10 sample tray, a programmable four gradient pump, a peltier column oven, a DAD and a UV/VIS detector. All parts were either directly or indirectly connected through NCI 900 (Network Chromatography Interface) interfaces to a Windows XP system, controlled with Turbochrom Navigator, Version 6.3.1. On this software also all further integration and calibration was taken out.

2.3 Samples

The samples were taken in accordance with the "Weinbauschule Klosterneuburg" from their viticulture research area in the premises of the secondary educational establishment for oenology. Crosses mark the sample rows:

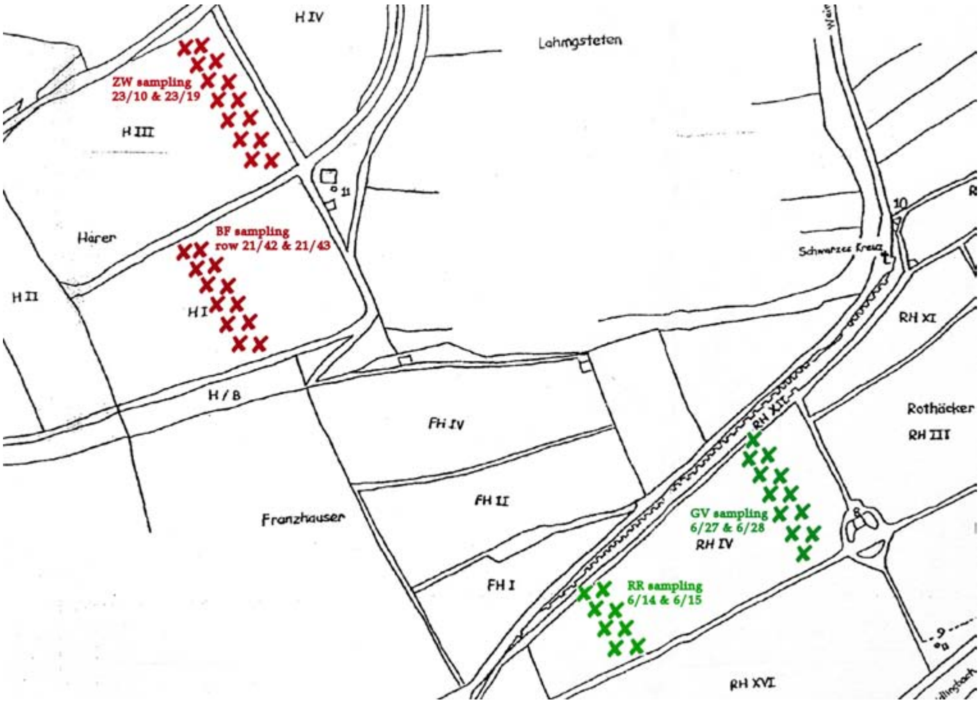


Figure 6: Samples taken at Weinbauschule Klosterneuburg

The whole area is separated into quarters:

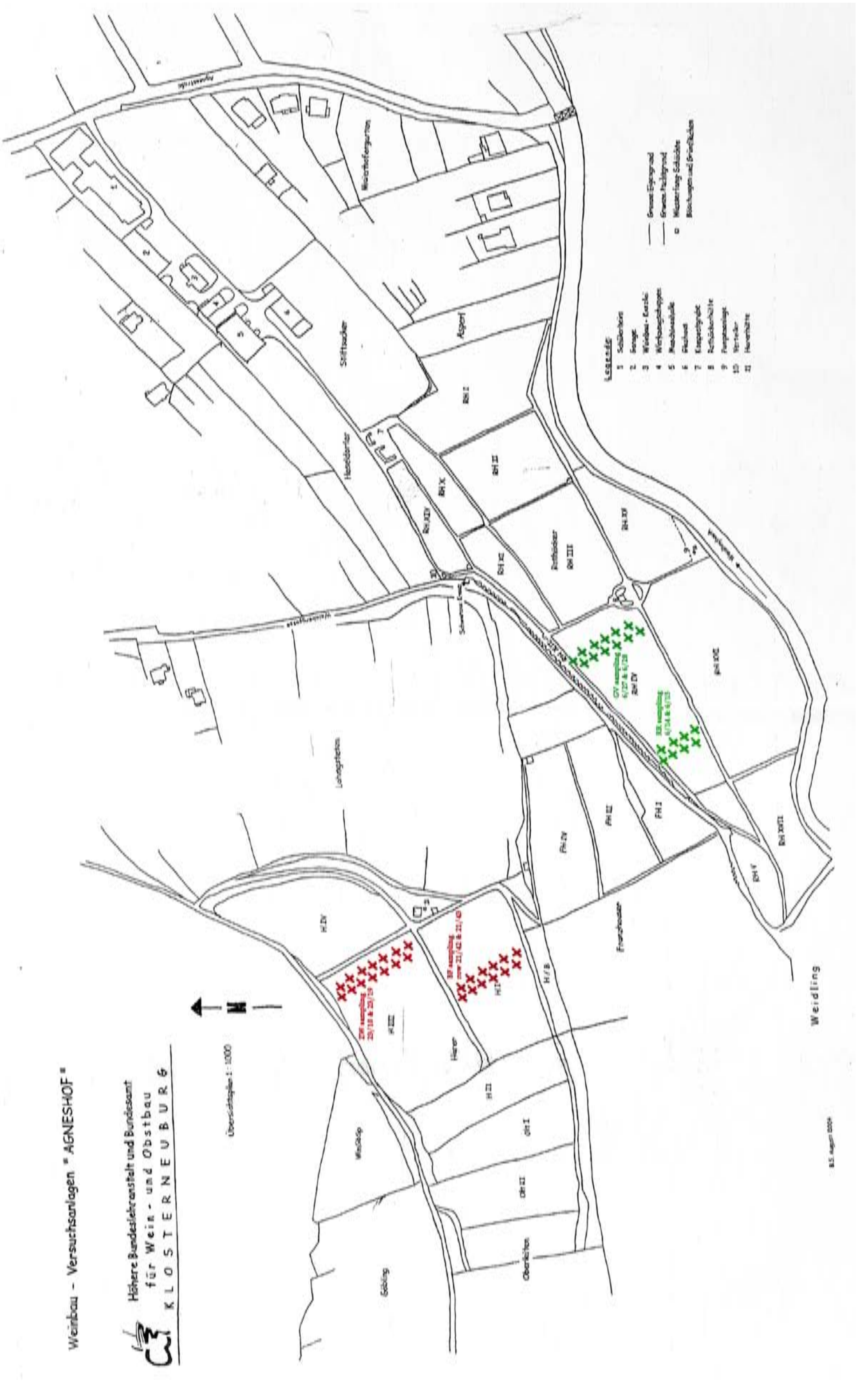


Figure 7: Weinbauschule Klosterneuburg Agneshof

The vines are all grown on grape phylloxera resistant rootstock. Commercial cultivars of grapevines are usually grafted on American vines like *Vitis riparia, Vitis rupestris* and *Vitis berlandieri* and crossings of these or hybrids with the European species *Vitis vinifera* and are given numbers or are combined with names. The samples are taken from the four most common grape cultivars in Austria and have following records:

- Blaufränkisch (BF): quarter H I; in row 21/42 cultivar clone M3 and row 21/43 cultivar clone 148; rootstock Fercal; bed out in 2002; machine track mulched

Figure 8: BF, row 21/43, July

- Grüner Veltliner (GV): quarter RH IV; row 6/27 and 6/28; clone 100; rootstock Kober 5BB; bed out 1992; treated with herbicides; machine track mulched

Figure 9: GV, row 6/28, July

- Rheinliesling (RR): quarter RH IV; row 6/14 and 6/15; clone 239 Geisenheim; rootstock Kober 5BB; bed out 1993; treated with herbicides; machine track mulched

Figure 10: RR, row 6/15, April, track is mulched

- Zweigelt (ZW): quarter H III; row 23/10 - rootstock 725, 779, 1103 Paulsen; row 23/19 - rootstock 8B, 125AA, R27; row 23/28 - rootstock G26, 41B, Fercal; row 23/37 - rootstock R7, Cosmo2, Cosmo10; bed out 1996; machine track mulched

Figure 11: ZW, row 23/19+28, after exfoliative means, September

The dates of sampling were set close to the usual time where exfoliative measures are taken out in the vine yards and are collected from the whole row and mixed afterwards, a representative sample for this cultivar in this quarter. Tendering vine in conventional cultivation includes application of all kinds of chemicals: pesticides, fungicides, insecticides and acaricides, sometimes even herbicides.

In 2009 there have been seven treatments in the following time schedule:

- 21st of April: 0,7% sulfur, outlet treatment at the swell of the buds against fungus and acarids
- 8th of May: 0,3% sulfur, 0,75% Delan® WG (dithianon) and 0,25% Reldan 2E (chloryrifos-methyl), a mixture of insecticides and fungicides
- 25th of May: Universalis (azoxystrobin and folpet), a broadband fungicide
- 9th of June: Talendo® (proquinazid), Aktuan® (cymoxanil and dithianon) and Confidor® 70 WG (imidacloprid), fungicides and insecticides
- 3rd of July: Vivando® (metrafenone) and Ridomil® Gold Combi (metalaxyl-m and folpet), fungicides
- 14th of July: Aktuan®, Karathane® Gold (meptyldinocap) and Frupica® (mepanipyrim), fungicides

- 7th of August: Cuprozin® liquid (copper hydroxide), Cantus® (boscalid) and Topas® (penconazole), fungicides

The samples for this work were taken on:

- 23rd of April
- 4th of July and
- 24th of September

Figure 12: Sampling

Character	stems April 09		leaves July 09	leaves Sept 09	shoots July 09	shoots Sept 09	stems July 09	stems Sept 09
Sample Name	BF1	RR1	BF7	BF10	BF14	BF17	BF18	BF19
	BF2	RR2	BF8	BF11	BF15	GV16	GV18	BF20
	BF3	RR3	BF9	BF12	BF16	RR16	GV19	BF21
	BF4	RR4	GV7	BF13	GV14	ZW17	GV23	GV20
	BF5	RR5	GV8	GV10	GV15		RR18	GV21
	BF6	RR6	GV9	GV11	GV17		RR19	GV22
	GV1	ZW1	RR7	GV12	RR14		RR23	RR20
	GV2	ZW2	RR8	GV13	RR15		ZW18	RR21
	GV3	ZW3	RR9	RR10	RR17		ZW19	RR22
	GV4	ZW4	ZW7	RR11	ZW14		ZW20	ZW21
	GV5	ZW5	ZW8	RR12	ZW15			ZW22
	GV6	ZW6	ZW9	RR13	ZW16			ZW23
				ZW10				
				ZW11				
				ZW12				
				ZW13				

Table 1: Sample character

Differences could be found while taking samples and afterwards also in the proceedings. Some of the leaves appeared infected and after grinding left a tomentum.

Figure 13: GV, affected, September

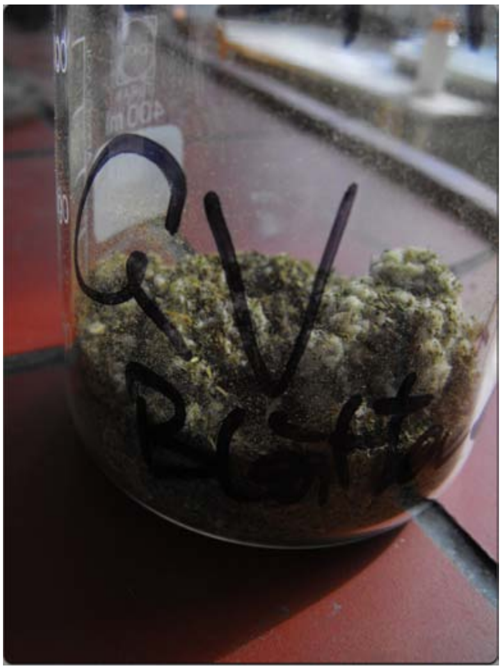


Figure 14: GV affected sample, with tomentum, after grinding

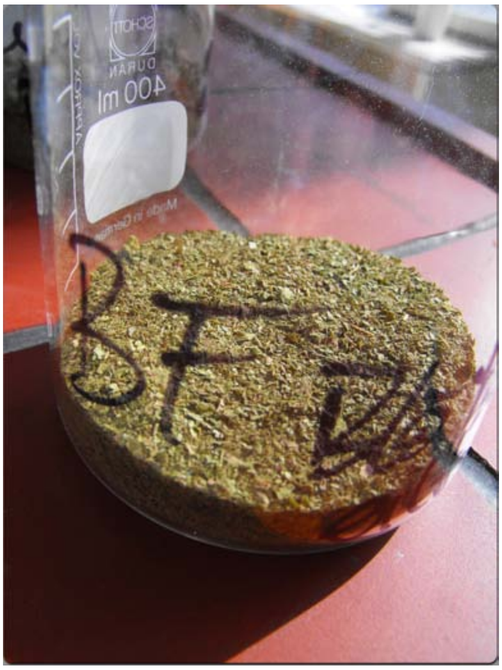


Figure 15: BF sample, normal, after grinding

3 Methods

3.1 First approach - OPC assay

The start of the work took place in the winter of 2009 and the late spring delayed the developing of vines as well as the connection to the Eubio network[53] for ordering standards for chromatography purposes. There was time enough to analyse the general amounts of proanthocyanidins in grape seeds, branches and leaves quantitavely. The sample of branches and leaves that were collected in November 2008 from the “Weinbauschule Klosterneuburg” for the first approach was “Blauer Zweigelt”. The seeds came from Weinviertel and there was a comparison between white and red grapes, “Grüner Veltliner” for white and “Blauer Zweigelt” for red samples. The grape seed oil was already pressed, and the analysis was done on the press cakes and the oil itself. The measurement was taken out with the vanillin assay[66]. In this method vanillin reacts through condensation with one of the aromatic rings in a specific position [67].

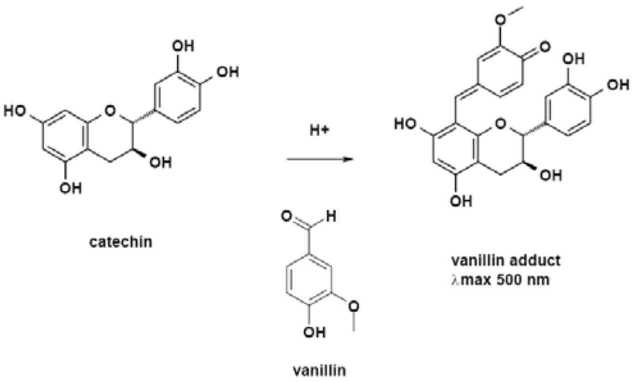


Figure 16: Vanillin reaction

This method requires analysis with the photometer at 500 nm and calibration with catechin. H_2SO_4 was used, which is claimed to being more sensitive than HCl[65]. The extraction was taken out overnight with EtOH/H_2O=70:30 as solvent. Afterwards the solvent mixture evaporated, the extract was weighed and taken up in the right dilution with methanol.
The calibration method included correction with three more factors:

To 1 mL of CT-Solution (0-300 µg/mL in methanol) or test solution in a test tube, 2,5 mL of methanol (control) or 1% vanillin solution in methanol (sample) and 2,5 mL of 9N H_2SO_4in methanol was added. The reaction mixture was incubated for 20 min at 30°C and the absorbance at 500 nm was measured. A was calculated as follows for each standard and sample solution:

A=$(A_s - A_b)$-$(A_c - A_0)$

A_s**:** Sample [1 mL test solution + 2,5 mL vanillin solution + 2,5 mL H_2SO_4]

A_s**:** Sample of 0 mg CT [1 mL methanol + 2,5 mL vanillin solution + 2,5 mL H_2SO_4]

A_c**:** Control [1 mL test solution + 2,5 mL methanol + 2,5 mL H_2SO_4]

A_0**:** Control of 0 mg CT [1 mL methanol + 2,5 mL methanol + 2,5 mL H_2SO_4]

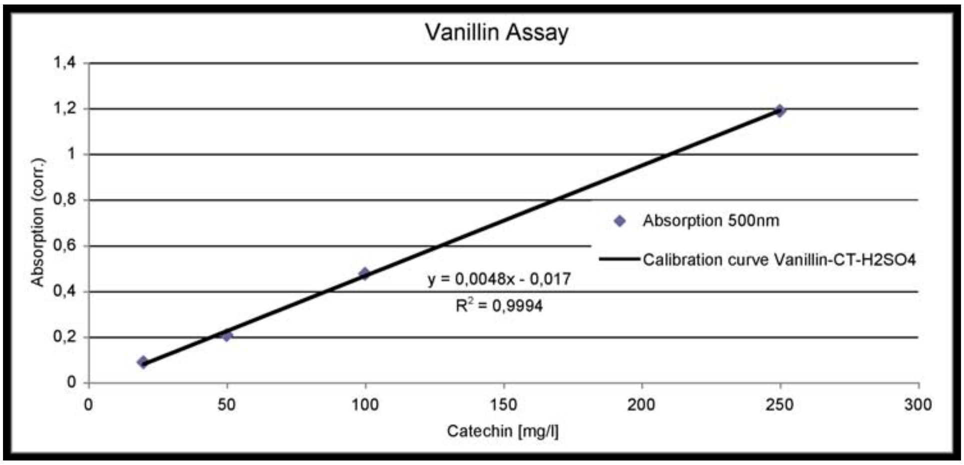


Figure 17: Vanillin calibration

3.2 Standards

2 mg of trans-resveratrol and piceid were weighed into a 20 mL volumetric flask to obtain a first stock solution. It was just used for spiking and after dilution the other concentration of 5 µg/mL for testing the HPLC. The resveratrol itself and its solutions were stored in the freezer at -20°C, the piceid at 4°C.

Later 0,05 g of pure resveratrol was weighed into a 50 ml volumetric flask for obtaining a stock solution, which was used for spiking and the dilution series was used for standard calibration.

desired concentration [mg/ml]	dilution of stock solution (in volumetric flasks)	required amount [ml]
0,05	1:2 (10 ml vol.flask)	5
0,02	1:5 (5 ml vol.flask)	1
0,01	1:10 (5 ml vol.flask)	0,5
0,005	1:20 (10 ml vol.flask)	0,5

Table 2: Dilution series resveratrol

3.3 Extraction

Samples were air dried in the absence of light in a ventilated and separated room. They were ground for 90 seconds in the mill.

Figure 18: Grinding samples, A: RR leaves Sept., B: GV leaves Sept., C: GV leaves Sept. grinded

Afterwards the extraction was made in the shaking bath at 60 rotations per minute and 25°C for 72 hours. Methanol was used as a solvent in a 1:10 (w/v) ratio. Many of the samples were spiked for recovery values with the stock solution (0,1 mg/ml) of the resveratrol standard.

Sample Name	Weight [g]	Dilution [ml]	Spiking [ml]	Sample Name	Weight [g]	Dilution [ml]	Spiking [ml]
GV1	2,0594	25	0,04	RR1	2,0517	25	0,025
GV2	2,0754	25	0,05	RR2	2,0593	25	0,075
GV3	2,061	25	0,1	RR3	2,0527	25	0,15
GV4	2,0656	25	0,25	RR4	2,0593	25	0,5
GV5	2,0615	25		RR5	2,057	25	
GV6	2,0762	25		RR6	2,0531	25	
GV7	2,1413	20		RR7	2,0561	20	
GV8	2,078	20		RR8	2,0141	20	
GV9	2,0467	20	5	RR9	2,0942	20	0,1
GV10	2,103	20		RR10	2,0821	20	
GV11	2,0316	20		RR11	2,0245	25	
GV12	2,0933	20	2,5	RR12	2,0673	20	0,25
GV13	2,0903	20	5	RR13	2,0747	20	0,5
GV14	2,0372	20		RR14	2,0178	20	
GV15	2,0347	20		RR15	2,0302	20	
GV16	0,7013	20		RR16	1,0282	20	
GV17	0,7554	20	0,245	RR17	1,4529	20	0,3
GV18	2,1256	20		RR18	2,1056	20	
GV19	2,0414	20		RR19	2,0591	20	
GV20	2,1071	20		RR20	2,0819	20	
GV21	2,0584	20		RR21	2,1481	20	
GV22	2,1546	20	0,8	RR22	2,03	20	1,2
GV23	2,1256	20	2	RR23	2,0714	20	1,5

Table 3: White cultivars weight and spike

Sample Name	Weight [g]	Dilution [ml]	Spiking [ml]	Sample Name	Weight [g]	Dilution [ml]	Spiking [ml]
BF1	2,0565	25	0,04	ZW1	2,0562	25	0,025
BF2	2,0537	25	0,075	ZW2	2,0601	25	0,05
BF3	2,0526	25	0,1	ZW3	2,0546	25	0,15
BF4	2,0528	25	0,5	ZW4	2,054	25	0,25
BF5	2,0529	25		ZW5	2,0596	25	
BF6	2,0525	25		ZW6	2,0559	25	
BF7	2,0643	20		ZW7	2,0437	20	
BF8	2,1028	20		ZW8	2,1042	20	
BF9	2,0679	20	0,25	ZW9	2,0387	20	0,1
BF10	2,038	20		ZW10	2,0306	20	
BF11	2,0197	20		ZW11	2,0357	25	
BF12	2,1205	20	1	ZW12	2,0289	25	0,5
BF13	2,0866	20	2,5	ZW13	2,1476	20	1
BF14	2,0094	20		ZW14	2,0665	20	
BF15	2,0368	20		ZW15	2,0248	20	
BF16	1,4402	20		ZW16	2,02	20	0,7
BF17	0,6783	20		ZW17	0,6732	20	
BF18	2,0139	20		ZW18	2,1015	20	
BF19	2,1256	20		ZW19	2,0328	20	
BF20	2,1776	20		ZW20	2,0148	20	0,8
BF21	2,0414	20	2	ZW21	2,0916	20	
				ZW22	2,0958	20	1,5
				ZW23	2,1664	20	1,5

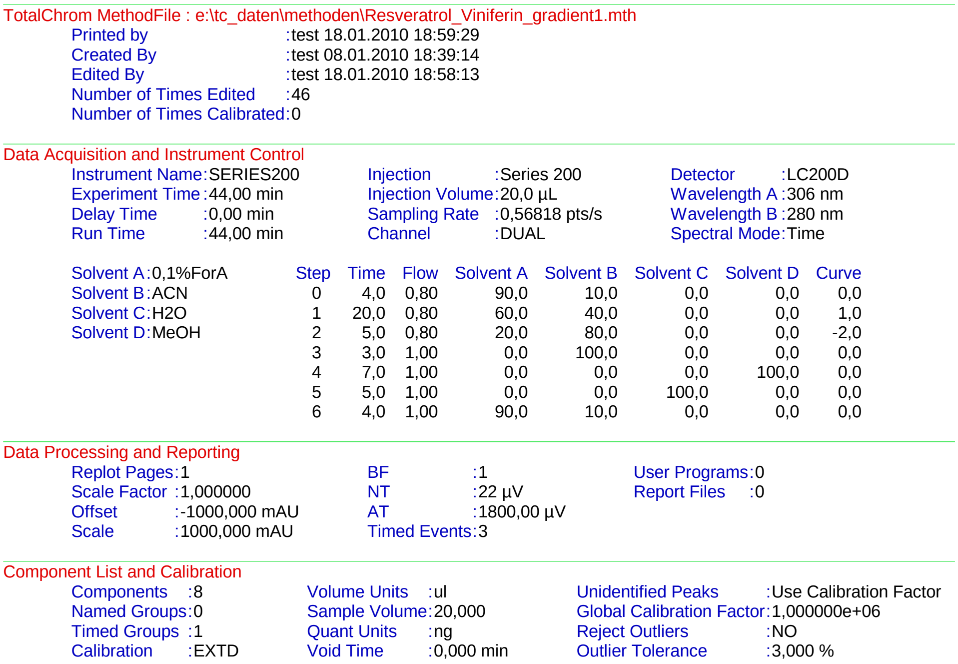
Table 4: Red cultivars weight and spike

3.4 Photometric analysis

Some representative samples were taken for a broad overview of absorbing characteristics and compared to the spectra of the standards. Since they were diluted in methanol, absorption could not be measured below 200 nm. The UV/VIS spectra were recorded for 200 to 800 nm, either the undiluted sample, at 1:10 and 1:100, regarding the absorption intensity. The visible wavelengths were meant to be used for color analysis, since the methanolic solution showed different colour ranges for extracted leaves and stems.

3.5 HPLC analysis

A long procedure finally ended in a satisfying outcome. All possible failures needed to be eliminated before reasonable results could be obtained. The very certain specifications of the acquired samples and appropriate analysis required the developing of a new method. In the research work from Püssa et al [30] a gradient with acetonitrile and 0,1% formic acid was described. This method finally did not work out that well and so adjustments were taken out until sufficient separation of all compounds was accomplished. After three to four analyses in a row, the retention time shifted and regeneration of the column had to be carried out. For gaining the best results possible, flushing with methanol and water after every run was undertaken to surely get clean and reproducible peaks.



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TotalChrom MethodFile : e:\tc_daten\methoden\Resveratrol_Viniferin_gradient1.mth
          Printed by                   :test 18.01.2010 18:59:29
          Created By                   :test 08.01.2010 18:39:14
          Edited By                    :test 18.01.2010 18:58:13
          Number of Times Edited       :46
          Number of Times Calibrated:0

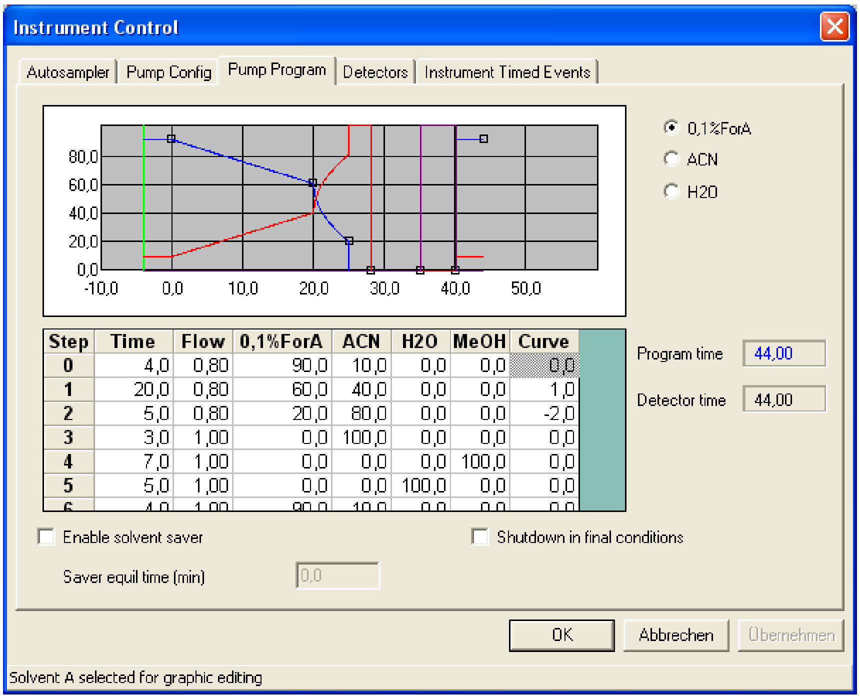
Data Acquisition and Instrument Control
          Instrument Name:SERIES200         Injection        :Series 200        Detector       :LC200D
          Experiment Time :44,00 min        Injection Volume:20,0 µL            Wavelength A :306 nm
          Delay Time       :0,00 min        Sampling Rate    :0,56818 pts/s     Wavelength B :280 nm
          Run Time         :44,00 min       Channel          :DUAL              Spectral Mode:Time

          Solvent A:0,1%ForA          Step  Time   Flow  Solvent A  Solvent B  Solvent C  Solvent D  Curve
          Solvent B:ACN                  0   4,0   0,80       90,0       10,0        0,0        0,0    0,0
          Solvent C:H2O                  1  20,0   0,80       60,0       40,0        0,0        0,0    1,0
          Solvent D:MeOH                 2   5,0   0,80       20,0       80,0        0,0        0,0   -2,0
                                         3   3,0   1,00        0,0      100,0        0,0        0,0    0,0
                                         4   7,0   1,00        0,0        0,0        0,0      100,0    0,0
                                         5   5,0   1,00        0,0        0,0      100,0        0,0    0,0
                                         6   4,0   1,00       90,0       10,0        0,0        0,0    0,0

Data Processing and Reporting
          Replot Pages:1                    BF            :1               User Programs:0
          Scale Factor :1,000000            NT            :22 µV           Report Files   :0
          Offset        :-1000,000 mAU      AT            :1800,00 µV
          Scale         :1000,000 mAU       Timed Events:3

Component List and Calibration
          Components    :8          Volume Units   :ul           Unidentified Peaks        :Use Calibration Factor
          Named Groups:0            Sample Volume:20,000         Global Calibration Factor:1,000000e+06
          Timed Groups  :1          Quant Units    :ng           Reject Outliers           :NO
          Calibration   :EXTD       Void Time      :0,000 min    Outlier Tolerance         :3,000 %
```

Figure 19: Conditions for HPLC information gathering



● 0,1%ForA
○ ACN
○ H2O

Step	Time	Flow	0,1%ForA	ACN	H2O	MeOH	Curve
0	4,0	0,80	90,0	10,0	0,0	0,0	0,0
1	20,0	0,80	60,0	40,0	0,0	0,0	1,0
2	5,0	0,80	20,0	80,0	0,0	0,0	-2,0
3	3,0	1,00	0,0	100,0	0,0	0,0	0,0
4	7,0	1,00	0,0	0,0	0,0	100,0	0,0
5	5,0	1,00	0,0	0,0	100,0	0,0	0,0
6	4,0	1,00	90,0	10,0	0,0	0,0	0,0

Program time 44,00

Detector time 44,00

☐ Enable solvent saver

☐ Shutdown in final conditions

Saver equil time (min) 0,0

Solvent A selected for graphic editing

Figure 20: Pump programme

Step	Time	Flow	0,1% formic acid	acetonitrile	water	methanol	Curve
	-4	0,8	90	10	0	0	0
0	0	0,8	90	10	0	0	0
1	20	0,8	60	40	0	0	1
2	25	0,8	20	80	0	0	-2
3	28	1	0	100	0	0	0
4	35	1	0	0	0	100	0
5	40	1	0	0	100	0	0
6	44	1	90	10	0	0	0

Table 5: HPLC pump parameters

With this method the peaks are well distinguished from one another. They do not show any kind of problematic tailing or fronting nor is the baseline disrupted with absorbing solvents. The system peaks interfering with automatic peak detection were easily extinguished with three baseline timed events with opting in and out the automatic detection. Reasonable scaling starts therefore at 4 min and ends at 32 min. The flow rate at 0,8 mL/min was found while watching the pressure not to exceed 2000 PSI.

3.5.1 Data processing

TotalChrom® software stores the raw data on the hard disk, which can afterwards be re-integrated and re-processed graphically or batched with the updated method. During the peak detection step in data analysis, the software scans the data points in a raw data file (or modified raw data file) to find peaks and saves the data it collects in a result file. Several parameters define the baseline, which is of the utmost importance to distinguish between noise and peaks.

The bunching factor specifies how many sequential data points in a raw data file are grouped in a bunch. During peak detection, the bunches are counted and the voltage values of the points in each bunch are averaged. The resulting averages are assigned to bunched points.

The parameter for noise threshold uses the vertical difference between two different consecutive bunched data points to recognize the potential start of a peak. A higher noise threshold requires a more abrupt rise between data values before a peak start can be detected. The parameter for area threshold is used after the noise threshold to confirm the potential start of peaks that pass the noise threshold test. Pairs of bunched data points must continue to exceed the noise threshold, and the cumulative sum of the bunched data points on the leading edge must eventually exceed the area threshold for the peak to be confirmed. A good policy when starting out is to use a low noise threshold and a high area threshold. This maintains a high degree of sensitivity in detecting peak starting and ending points, but still screens out noise spikes.

Graphic peak separation was a bit delicate with two main values to be considered which affect the baseline drawn beneath the peaks. The Width Ratio is the ratio of the distance between the end of the first peak and the start of the second peak to the width of the second peak at its base. The Valley-to-Peak Ratio is the ratio of the height of the valley between peaks to the height of the smaller peak.

3.5.2 Resveratrol calibration

The calibration was made with linear regression for defined amounts injected into the system and the corresponding integrated areas give quite a satisfying trendline with good determination.

Sample Name	Amount [µg/ml]	injected amount [µg]	Area [µV·s]
Resv5µg_old	5	0,1	1,0032E+06
Resv5µg_newnew	5	0,1	8,9600E+05
Resv10µg_old	10	0,2	1,0089E+06
Resv10µg_newnew	10	0,2	1,0611E+06
Resv20µg_old	20	0,4	2,9775E+06
Resv20µg_new	20	0,4	2,7458E+06
Resv50µg_new	50	1	6,9433E+06
Resv50µg_newnew	50	1	6,8409E+06

Table 6: Resveratrol samples and integrated area

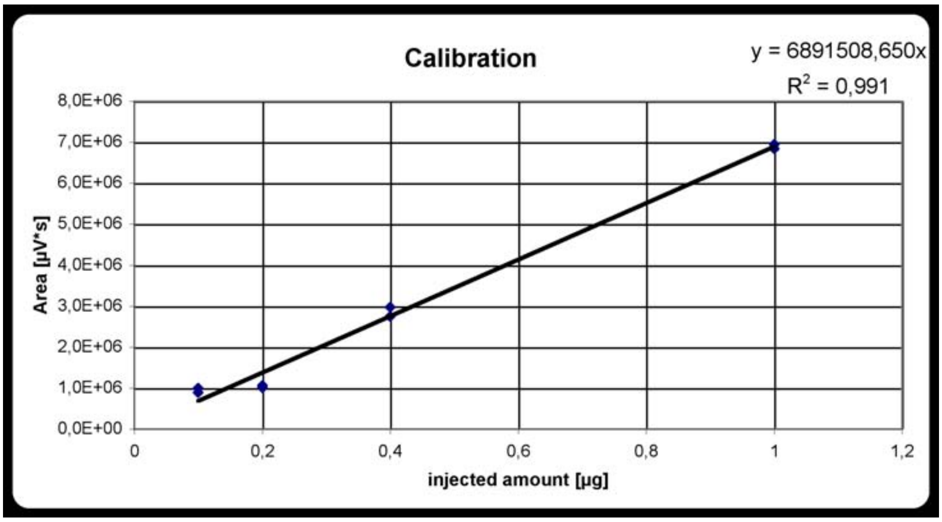


Figure 21: Resveratrol calibration

3.5.3 Stability

Degradation of resveratrol was checked within a period of half a year. Freshly purchased, weighed in and dissolved pure resveratrol-powder was compared to older standards, diluted in methanol of the same concentration. The stock solution was also analysed for activity loss.

Date	appendix given
04/22/2009	_old
10/15/2009	_new
11/03/2009	_newnew

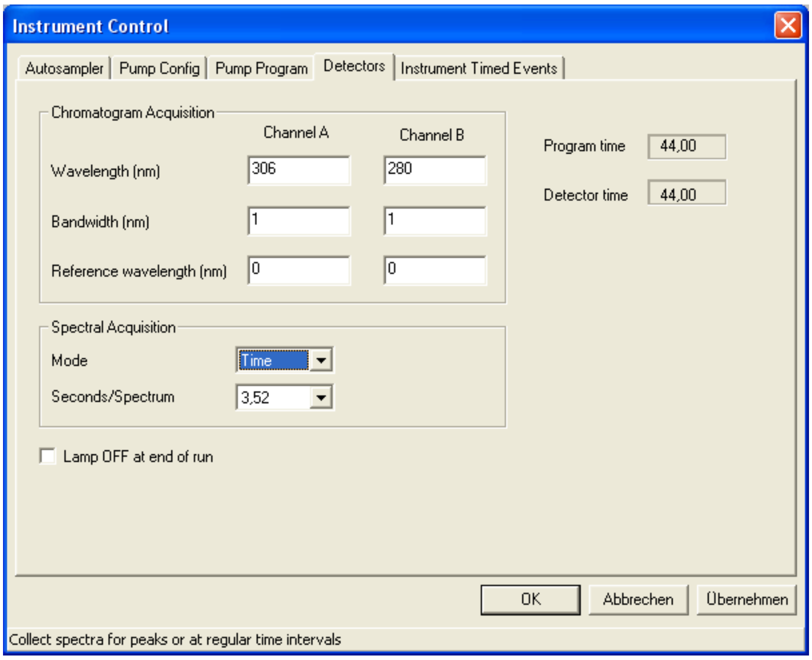
Table 7: Standard production

3.5.4 Recovery

42% of the samples were spiked with a certain amount of resveratrol stock solution to calculate recovery values. Therefore the calculated amount from the spiked samples where substracted from the non-spiked samples. These values were refered to the calculated spiked amount therein.

3.5.5 UV/VIS Detector, DAD

The maximum absorption of trans-resveratrol is at around 306 nm. So the DAD and the UV/VIS detector were set to measure at this wavelength. The second channel from the DAD also recorded at 280 nm, which is the maximum of flavanols. The bandwidth was set to 1 nm for extinguishing the disturbing absorption capacity of other components close to the desired wavelength. The DAD was used for recording whole spectra every 3,52 seconds ranging from 190 nm to 700 nm, so as to be able to distinguish the different components, whereas the UV/VIS detector was used for distinct analysis.



Chromatogram Acquisition

	Channel A	Channel B
Wavelength (nm)	306	280
Bandwidth (nm)	1	1
Reference wavelength (nm)	0	0

Program time 44,00

Detector time 44,00

Spectral Acquisition

Mode Time

Seconds/Spectrum 3,52

☐ Lamp OFF at end of run

Collect spectra for peaks or at regular time intervals

Figure 22: Detector settings

3.5.6 Spectral processing

The automated recording of UV/VIS spectra between 190 to 700 nm, saved by the DAD every three seconds, can be used for identification of designated compounds. With the external but linked software IRIS, spectral data can be viewed and manipulated. It is possible to compare the spectra of the standards with a sample at the same retention time to make sure it is the same component. Also the height of the peaks can be shown in numbers which gives an idea of the contained amount of the respective substance. According to literature [30], whenever almost similar samples were analysed with MS and verified with commercial standards, the retention time of reoccuring peaks was estimated and named in relation to the piceid and resveratrol standards as refering substances.

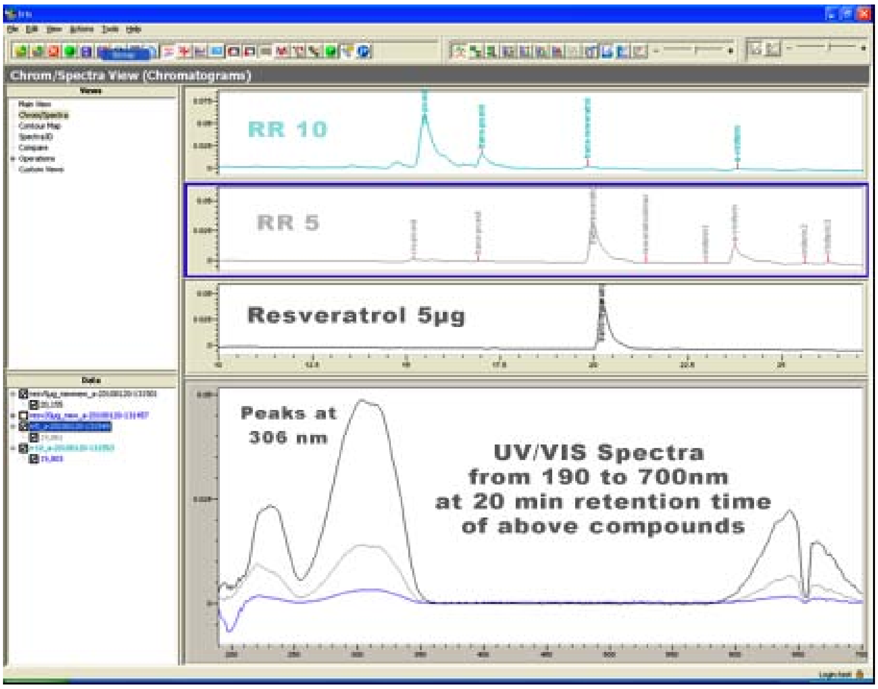


Figure 23: Resv5µg - RR5 - RR10

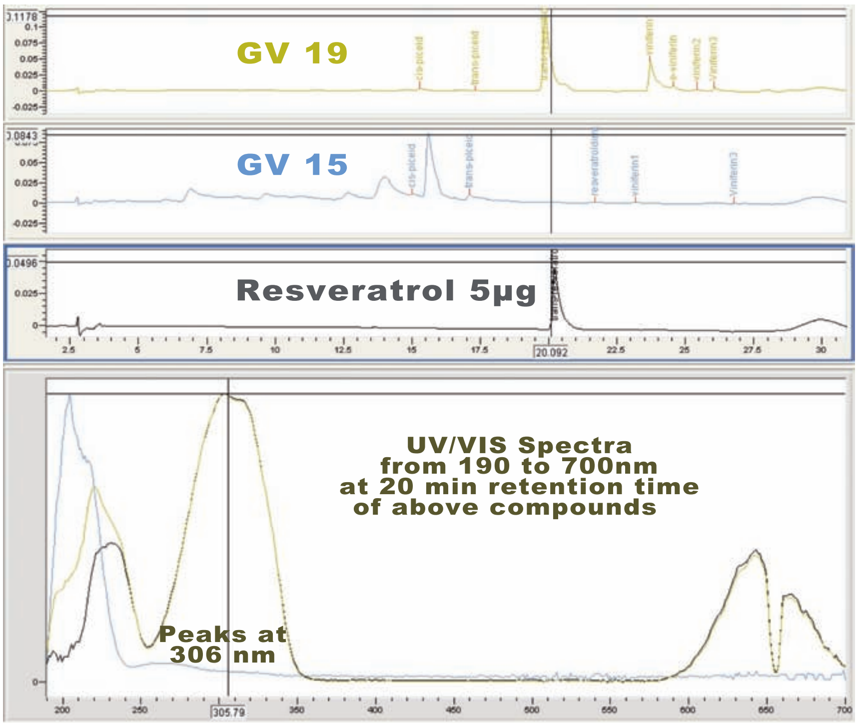


Figure 24: Resv5µg - GV15 - GV19

4 Results

4.1 OPC assay

These are the results of the measurement:

	Yield [%]	A	Catechin [mg/L]	Catechin Equ [w/w %]	OPC content [%]
Presscake GV	19,16	0,31	67,60	22,05	4,23
Presscake ZW	15,14	0,16	37,40	15,44	2,34
Grapeseedoil GV	3,20	0,28	61,98	0,19	0,01
Grapeseedoil ZW	8,44	0,25	56,15	0,06	0,01
Stems ZW	24,13	0,03	10,73	0,55	0,13
Leaves ZW	9,21	0,27	59,58	6,12	0,56

Table 8: Proanthocyanidin assay

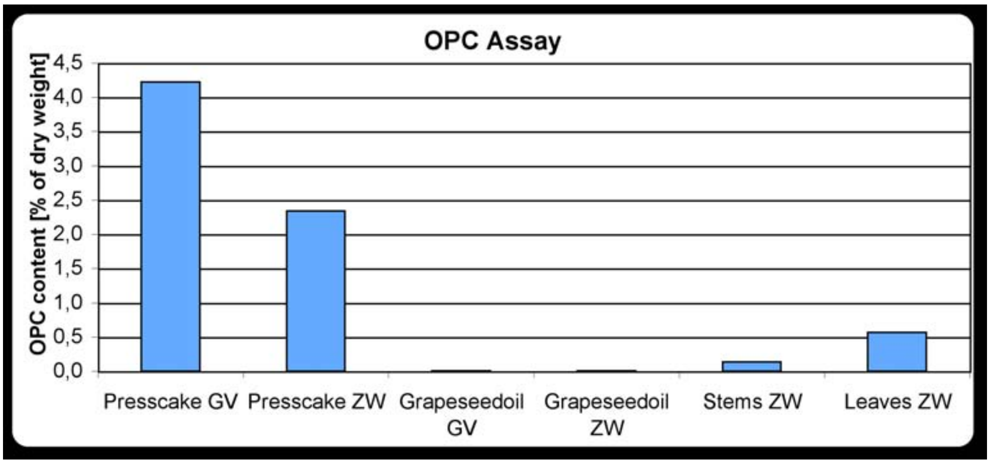


Figure 25: OPC assay

4.2 Photometric Analysis

The representative absorption spectra from standards and different samples are given in the following wavelength scans in different concentrations for UV and VIS.

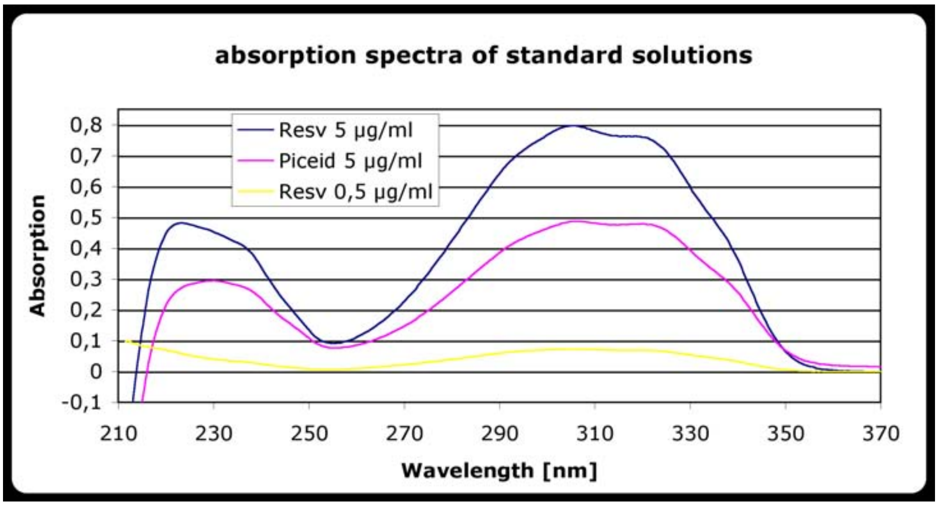


Figure 26: Absorption spectra of standards

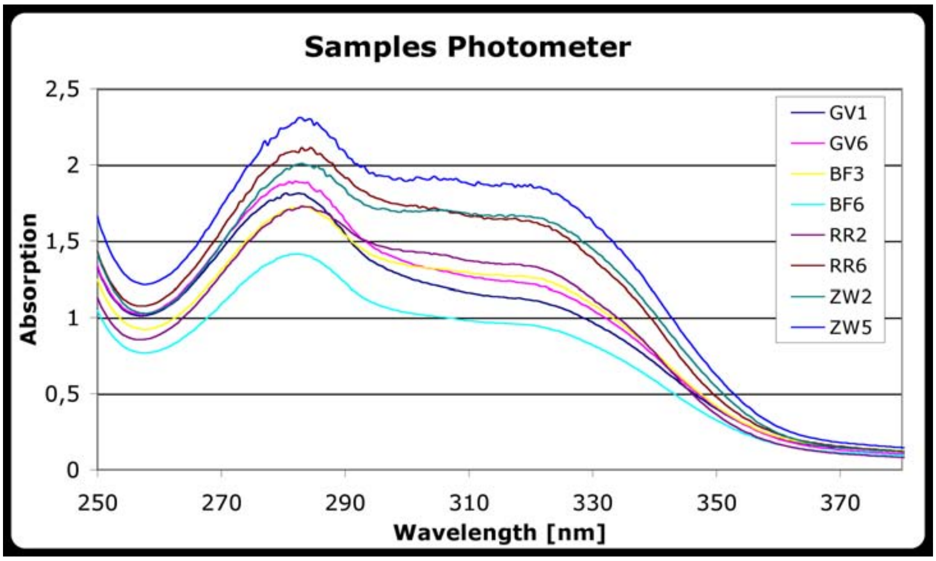


Figure 27: Absorption spectra stems April 2009 diluted 1:10

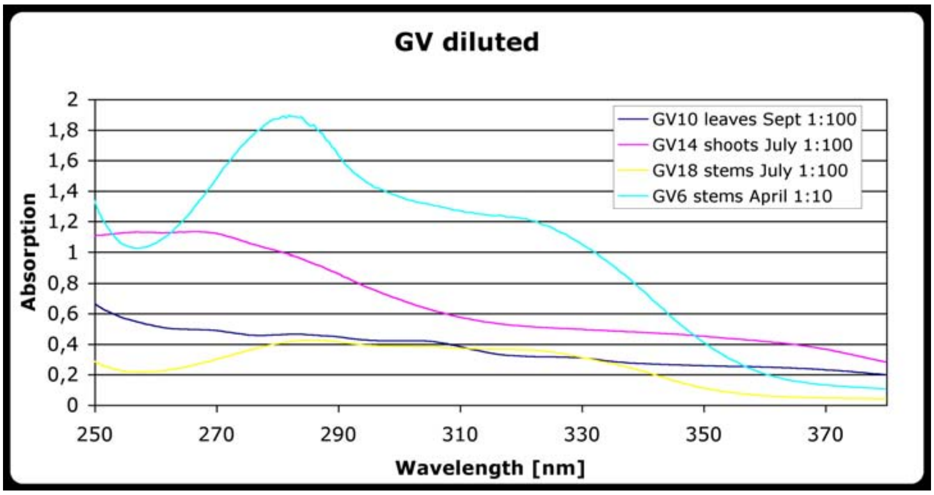


Figure 28: Absorption spectra GV diluted

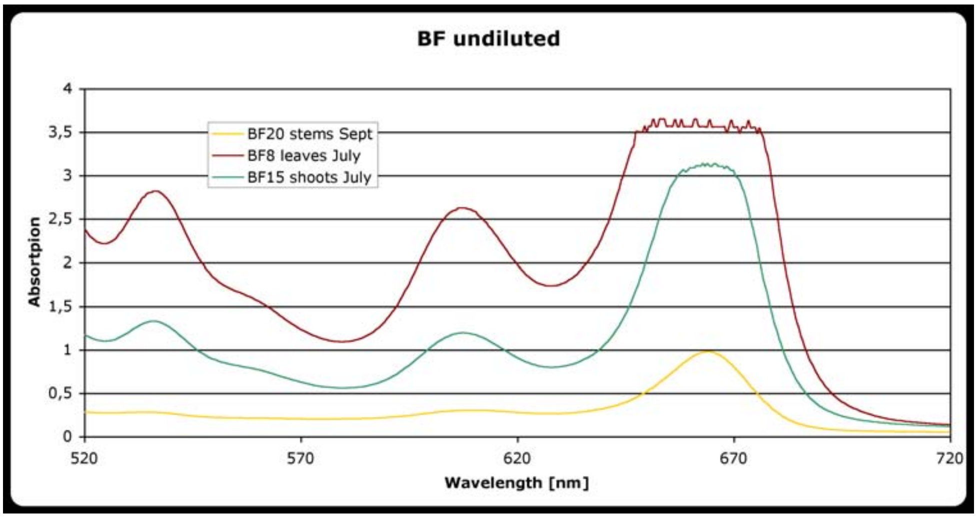


Figure 29: Absorption spectra BF undiluted

4.3 Evaluation

4.3.1 HPLC evaluation

The automated integration of the peaks at a certain time range, at around 20 minutes retention time, was accurate at 89% of all samples. In four cases, the integration line was corrected by hand and in seven more cases a factor was calculated for the height to area ratio of certain components whenever the automatically drawn line was distinguishing from the others of the same character.

An analysis comparison of the same sample measured at there different times show the accuracy of the HPLC method and the detection.

	14012010	18012010	26012010	**Deviation**		
components	Area [µV·s]	Area [µV·s]	Area [µV·s]	**Area [µV·s]**	**Amount [mg/g]**	**[%]**
cis-piceid	562433,4	427968,1	546166,7	11502,3	0,0041	2,2
cis-resveratrol	2217150,4	2029880,5	2166698,4	35675,0	0,0128	1,7
trans-piceid	258519,8	154122,4	249316,2	6508,0	0,0023	2,9
resveratrol-dimer	59940,1	60849,6	61511,1	1110,9	0,0004	1,8
viniferins	73446,1	72402,8	75340,7	1339,7	0,0005	1,8

Table 9: Comparison ZW15 determined three times

The shown components in this table represent the assignable components, although more peaks can be distinguished, see for example Figure 32 on page 44.

4.3.2 Qualitative and quantitative evaluation

In the following table, all the trans-resveratrol values of the integrated area and calculated amounts of the analysed samples are given, including spiked ones.

Sample Name	Character	Area [µV*s]	Amount [mg/g dw]	Sample Name	Character	Area [µV*s]	Amount [mg/g dw]
BF1*	st A	368908,5	0,1627	RR3*	st A	955430,0	0,4221
BF2*	st A	572098,1	0,2526	RR4*	st A	1243452,4	0,5476
BF3*	st A	547705,4	0,2420	RR5	st A	690090,4	0,3043
BF4*	st A	534121,9	0,2360	RR6	st A	639475,2	0,2825
BF5	st A	458431,0	0,2025	RR7	le J	27317,5	0,0096
BF6	st A	338207,0	0,1494	RR8	le J	30111,1	0,0108
BF7	le J	31840,1	0,0112	RR9*	le J	27299,1	0,0095
BF9*	le J	26033,9	0,0091	RR10	le S	54090,7	0,0188
BF10	le S	34820,1	0,0124	RR11	le S	44480,3	0,0199
BF11	le S	35917,4	0,0129	RR12*	le S	87428,0	0,0307
BF13*	le S	260523,2	0,0906	RR13*	le S	91021,0	0,0318
BF14	sh J	23889,5	0,0086	RR14	sh J	5356,4	0,0019
BF16	sh J	31687,4	0,0160	RR15	sh J	3038,8	0,0011
BF17	sh S	48219,0	0,0516	RR16	sh S	218844,7	0,1544
BF18	st J	2257036,6	0,8131	RR17*	sh J	54916,4	0,0274
BF19	st S	2018727,5	0,6891	RR18	st J	6356989,7	2,1904
BF20	st S	1517807,2	0,5057	RR19	st J	5277872,5	1,8597
BF21*	st S	1684777,24	0,5988	RR20	st S	3234377,3	1,1272
GV1*	st A	474916,61	0,2091	RR21	st S	3191053,4	1,0778
GV2*	st A	422117,26	0,1845	RR22*	st S	3613811,5	1,2916
GV3*	st A	334665,32	0,1473	RR23*	st J	6846685,7	2,3981
GV4*	st A	438164,20	0,1924	ZW3*	st A	790653,2	0,3490
GV5	st A	389585,46	0,1714	ZW4*	st A	936652,1	0,4136
GV6	st A	425913,24	0,1860	ZW5	st A	541290,3	0,2383
GV7	le J	150936,97	0,0511	ZW7	st A	26762,8	0,0095
GV9*	le J	35256,63	0,0125	ZW8	le J	19572,5	0,0067
GV10	le S	30091,88	0,0104	ZW9*	le J	11066,3	0,0039
GV11	le S	18243,79	0,0065	ZW10	le J	27593,4	0,0099
GV13*	le S	182961,36	0,0635	ZW11	le S	38205,1	0,0170
GV14	sh J	11743,07	0,0042	ZW12*	le S	42526,3	0,0190
GV15	sh J	10289,48	0,0037	ZW13*	le S	103581,6	0,0350
GV16	sh S	108275,12	0,1120	ZW16*	sh J	42322,3	0,0152
GV17*	sh J	70210,7	0,0674	ZW17	sh J	16493,2	0,0178
GV18	st J	2708313,9	0,9244	ZW18	sh S	2129262,7	0,7351
GV19	st J	2341379,7	0,8321	ZW19	st J	2001415,1	0,7143
GV20	st S	2347020,4	0,8081	ZW20*	st J	1732578,8	0,6239
GV21	st S	2162714,5	0,7623	ZW21	st J	2428902,8	0,8425
GV23*	st J	2665283,4	0,9097	ZW23*	st S	1990193,7	0,6665

Table 10: Single values for trans-resveratrol HPLC peaks
Spiked samples are marked with *.
For spiking concentrations refer to table 3 on page 29 and table 4 on page 30.

As a matter of fact the isomerization to other bioactive derivates takes place [subsection 4.5 on page 49], so the other amounts are part of the result as well. With the calibration from trans-resveratrol the amount of the other distinguishable components were determined as well. A comparison between the samples is reasonable for different specifications and also the period of the year the samples were taken.

In following trans-resveratrol amount figures, the scale is different according to every part of the plant. The trans-resveratrol amount in shoots is about one power of ten lower than the amount in stems. In leaves, the difference to stems is even to the second decimal power.

The tables show cis-resveratrol, trans resveratrol and viniferin amounts (including ε-viniferin and resveratrol-dimer).

4.3.2.1 Leaves The mass of compounds from the leaves of *Vitis vinifera* are generally early eluted with this chromatography method.

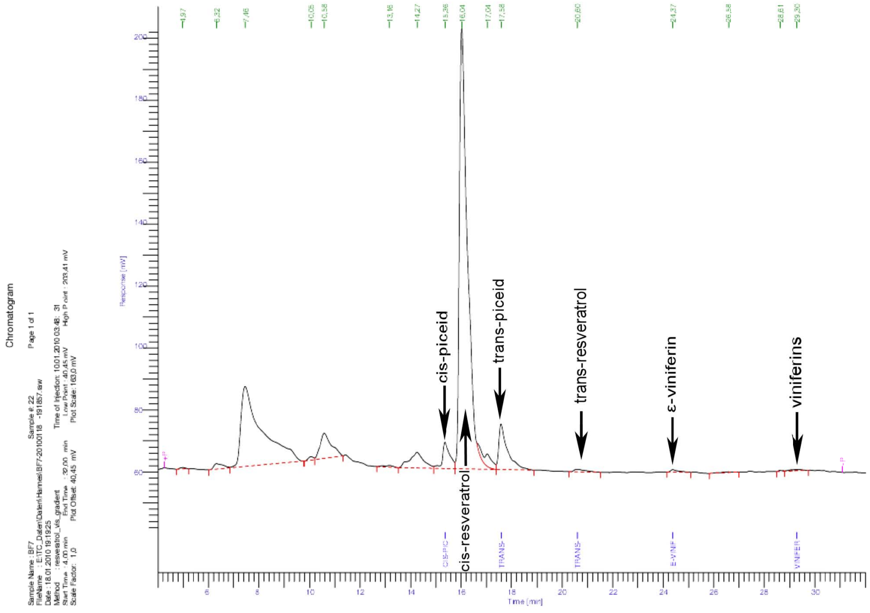


Figure 30: Chromatogram BF7 representing leaves

The first “TRANS” in the peak dedicated denotation refers to trans-piceid, the second “TRANS” around 20 min retention time is refering to the trans-resveratrol.

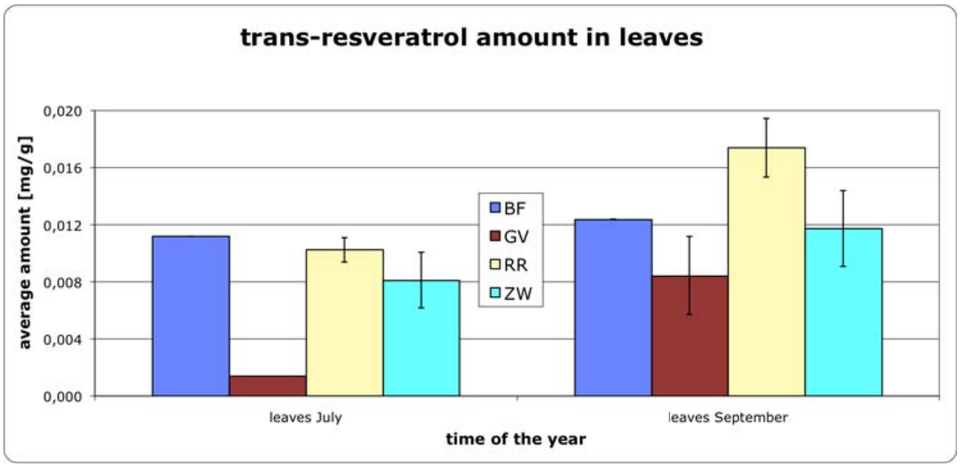


Figure 31: Resveratrol amount leaves

	cis-resveratrol [mg/g]	trans-resveratrol [mg/g]	viniferins [mg/g]
BF	1,0919	0,0112	0,0105
GV	0,6082	0,0511	0,0084
RR	0,6499 ± 0,0822	0,0102 ± 0,0009	0,0112 ± 0,0050
ZW	1,1381 ± 0,0135	0,0081 ± 0,0019	0,0063 ± 0,0011

Table 11: Amounts leaves July

	cis-resveratrol [mg/g]	trans-resveratrol [mg/g]	viniferins [mg/g]
BF	0,7237	0,0124	0,0037
GV	0,1680 ± 0,0130	0,0084 ± 0,0027	0,0087 ± 0,0004
RR	0,4604 ± 0,0175	0,0174 ± 0,0021	0,0099 ± 0,0005
ZW	0,7101 ± 0,0100	0,0117 ± 0,0027	0,0123 ± 0,0070

Table 12: Amounts leaves September

In all the leave samples from *Vitis vinifera* three main components could be distinguished. They are rich in cis-resveratrol between 0,2 mg/g dw to 1,2 mg/g dw in comparison to the amount of trans-resveratrol in these samples, at an order of one magnitude lower. The piceids can be identified at around 0,1 mg/g dw. There is also a not verified more hydrophilic component absorbing at that wavelength with around 7 minutes retention time, whose peak size is about the same or slightly higher than the trans-piceid in all the leave samples. It could involve catechin derivates.

4.3.2.2 Shoots The characteristic chromatogram of shoots show a variety of early eluted peaks.

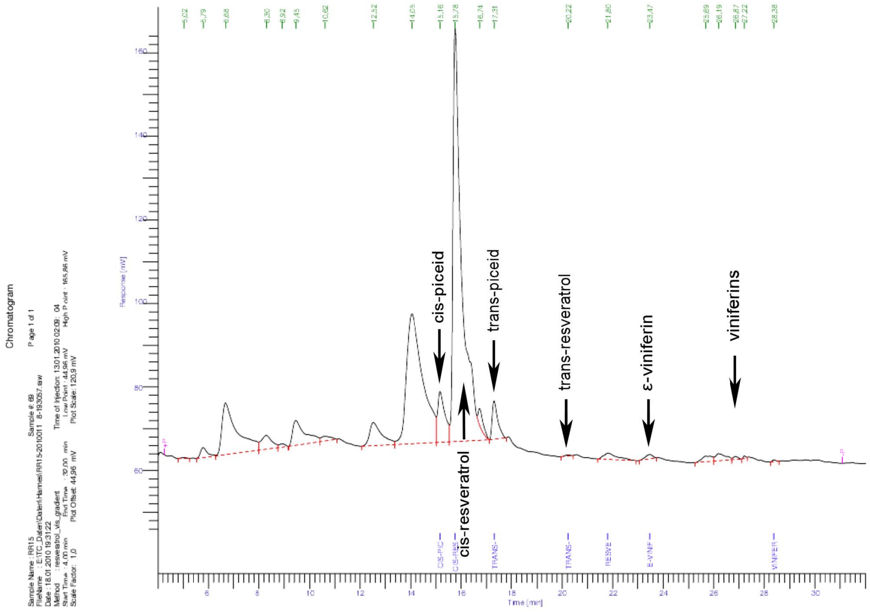


Figure 32: Chromatogram RR15 representing shoots

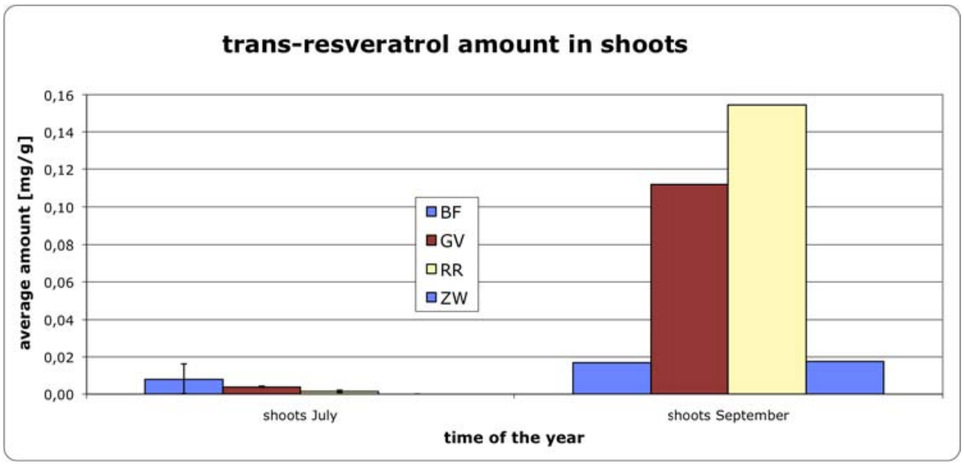


Figure 33: Resveratrol amount shoots

	cis-resveratrol [mg/g]	trans-resveratrol [mg/g]	viniferins [mg/g]
BF	1,0336 ± 0,4398	0,0123 ± 0,0052	0,0079 ± 0,0017
GV	0,7035 ± 0,0170	0,0039 ± 0,0004	0,0324 ± 0,0041
RR	0,8830 ± 0,0121	0,0015 ± 0,0006	0,0523 ± 0,0174
ZW	0,7944 ± 0,0949		0,0308 ± 0,0239

Table 13: Amounts shoots July

	cis-resveratrol [mg/g]	trans-resveratrol [mg/g]	viniferins [mg/g]
BF	0,2521	0,0169	0,0248
GV	0,3678	0,1120	0,0763
RR	0,1971	0,1544	0,1747
ZW	1,1355	0,0178	0,0516

Table 14: Amounts shoots September

The shoots vary in between the months the samples were taken. By trend the trans-resveratrol amount is higher in the more lignified parts taken in September. There are some more hydrophilic components that are not named. A big peak that appears in all the samples is the one right in front of the first named component. Generally cis-resveratrol is the leading component again in all the shoots ranging from 0,2 mg/g dw to 1,1 mg/g dw. The trend to earlier eluted components can be identified. In two samples the trans-resveratrol amount is quite high and only the ZW samples in July show no amplitude for trans-resveratrol.

4.3.2.3 Stems Only stems really synthesize bigger molecules in higher quantities.

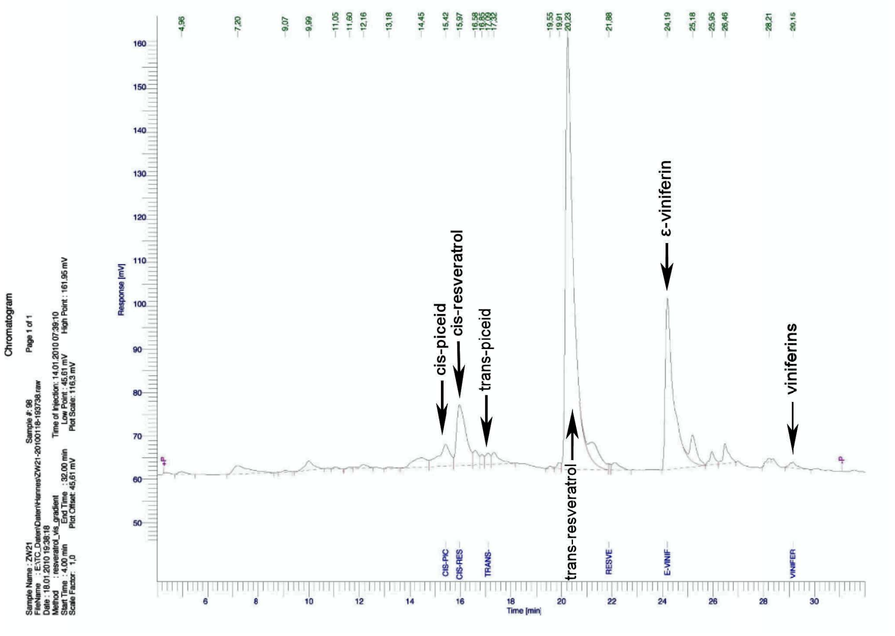


Figure 34: Chromatogram ZW21 representing stems

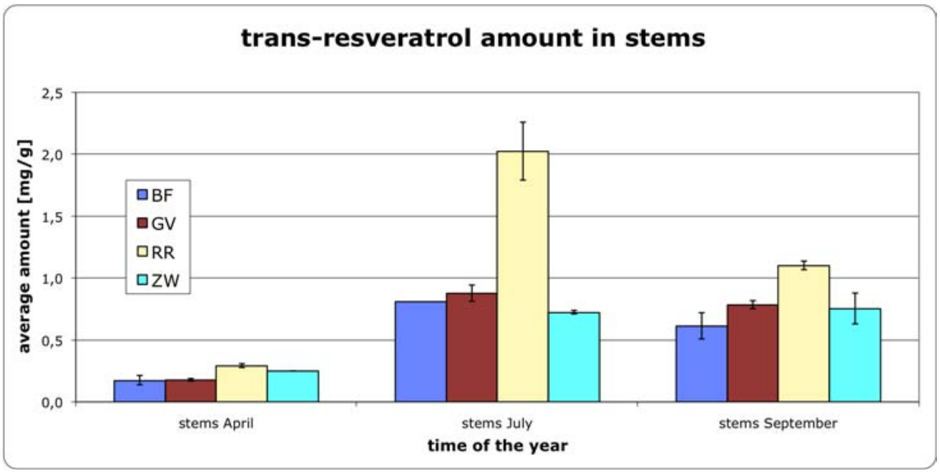


Figure 35: Resveratrol amount stems

	cis-resveratrol [mg/g]	trans-resveratrol [mg/g]	viniferins [mg/g]
BF	0,0048 ± 0,0016	0,1760 ± 0,0375	0,2420 ± 0,0543
GV	0,0074 ± 0,0001	0,1787 ± 0,0104	0,1724 ± 0,0234
RR	0,0109 ± 0,0000	0,2934 ± 0,0154	0,1470 ± 0,0089
ZW	0,0168 ± 0,0002	0,2508 ± 0,0177	0,4469 ± 0,0812

Table 15: Amounts stems April

	cis-resveratrol [mg/g]	trans-resveratrol [mg/g]	viniferins [mg/g]
BF	0,0537	0,8131	0,6860
GV	0,0241 ± 0,0073	0,8783 ± 0,0653	0,3714 ± 0,0647
RR	0,0340 ± 0,0390	2,0251 ± 0,2339	0,5403 ± 0,2109
ZW	0,0107 ± 0,0002	0,7247 ± 0,0147	0,8893 ± 0,1325

Table 16: Amounts stems July

	cis-resveratrol [mg/g]	trans-resveratrol [mg/g]	viniferins [mg/g]
BF	0,0844 ± 0,0093	0,6142 ± 0,1058	0,1700 ± 0,0361
GV	0,0489 ± 0,0074	0,7852 ± 0,0324	0,2438 ± 0,6768
RR	0,0510 ± 0,0044	1,1025 ± 0,0349	0,6971 ± 0,7288
ZW	0,0866 ± 0,0519	0,7545 ± 0,1245	0,3684 ± 0,0561

Table 17: Amounts stems September

The stems do not only have the highest general amount of trans-resveratrol but also provided most of the samples of this work, since the probing started before the developing of the first leaves and sprouts. Moreover, this is the main material that is drastically cut in late winter times and provides a lot of mulch material that does not rot very fast.

In the vegetative period of the plant, trans-resveratrol is isomerized in small quantities to cis-resveratrol, at around 0,03 mg/g dw. The amount is higher in later stages of the plant in relation to the total quantity and reaches tops of almost 0,1 mg/g dw. The piceids can also be found in all samples, usually more cis than trans-piceid. However, trans-resveratrol can be found in absolutely and relatively highest amounts through all the samples. They show highest output in July, were stress seems to be biggest. The peak area for this component together with the viniferins covers more than 90%, which states a dominant occurrence of oligomerizing enzymes and the plant's need to defend itself more at this time of the year.

4.3.3 Comparison cultivars

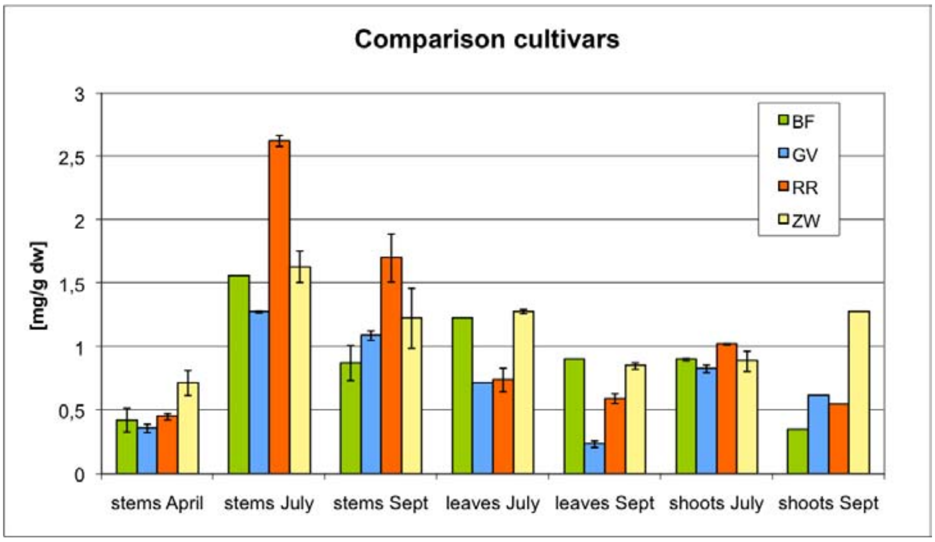


Figure 36: Comparison cultivars

The sum of cis- and trans resveratrol as well as the trans-piceid and the viniferins as identified peaks were calculated and are shown in the figure above. Generally the two dark varieties show bigger amounts of this category of polyphenols. Taking cis-resveratrol into account, leaves do not show any more far smaller amounts of these bioactive compounds.

In general the sum of the amounts are biggest in July. The old stems after the winter in contrast show the smallest quantities.

4.4 Stability

The chromatograms of older and newer standard solutions are compared.

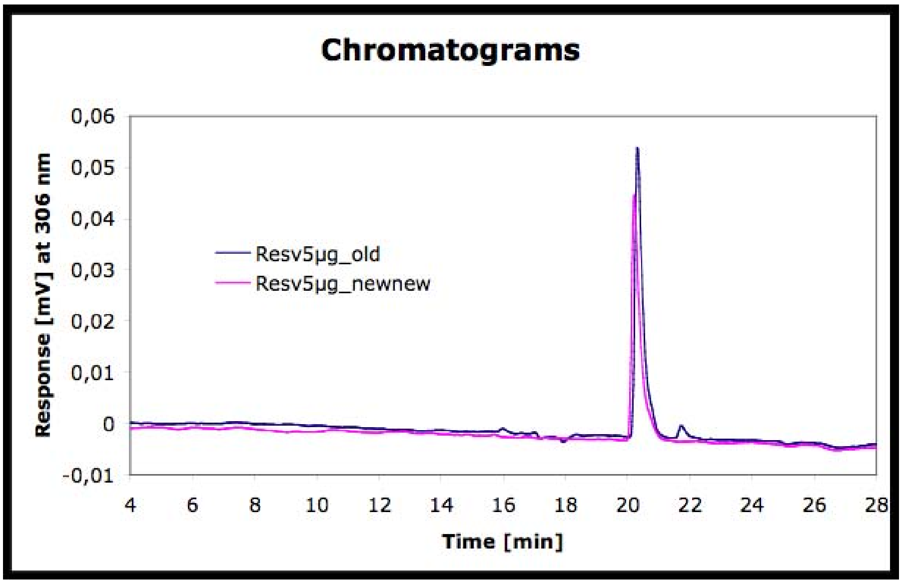


Figure 37: Comparison 5µg old and new

There is a slight decay after half a year when the are being kept in brown flasks in the freezer. Literature claims that irradiation with UV-light leads to an isomerization to the cis-isomer, which seems to occur in small amounts, before the peak. It can almost be recognized as a little heavier noise, taking around 2% of the whole integrable area. The slightly higher peak after the main peak signifies an oxidative product of trans-resveratrol; a viniferin, covering 11% of the area in comparison to the _*newnew* sample where the cis part covers 0,4% and the viniferins 4%.

4.5 Recovery

The adding of certain trans-resveratrol amounts to the sample before extraction started made an isomerization possible. Therefore the sum of possible immediate isomerization products was the only possible way to make recovery values visible.

character	sample name	spike concentration in sample [mg/g dw]	recovery [mg/g]	percentual recovery [%]
stems	BF	0,0871	0,0444	71,4
	GV	0,0533	0,0396	90,2
	RR	0,1580	0,1958	134,8
	ZW	0,0974	0,1355	126,9
leaves	BF	0,0121	0,0194	100,3
	GV	0,2443	0,2121	86,8
	RR	0,0137	0,0139	77,6
	ZW	0,0254	0,0046	93,6
shoots	GV	0,0324	0,0249	76,8
	RR	0,0206	0,0148	71,8
	ZW	0,0347	0,0293	84,6

Table 18: Recovery values
Recovery was calculated with the values for trans-resveratrol, cis-resveratrol, trans-piceid and the viniferins. The values for the supposed cis-piceids were only added to the calculations of shoots, since the impact on this material seemed to play a more significant role.

Following general aspects can be said:

- The highest spiking concentration was performed with the adding of 5 ml stock solution (0,1 mg /ml) which leads to an amount of up to 0,24 mg/g dw of pure trans-resveratrol. The smallest spiking concentration was done with 0,1 ml stock solution, which equals 0,005 mg/g dw.
- Small quantities easily lead to more distortion of the measured amount.
- Due to the inhomogeneity of the plant material, recovery values partly show quite high fluctuations that average values were formed.
- Many findings show percentages above 100%, which are speculatively due to a higher biosynthesis by the plant, once an enzymatic mechanism after spiking is activated.
- There is a high probability that within the three days of extraction the foil closures of the flasks were not equally sealed and methanol evaporated unevenly. When pouring the liquids and filtering the material, different amounts of liquids may also stick to the leftovers, which will lead again to inaccurate results. This error may sum up to almost 30% in worst cases.
- When adding the spiking solution to the dried and crushed bio-matter, an isomerization takes place enzymatically [71]. Moreover within the extraction procedure, filtering and refilling the sample solution in the small

flasks for analysing, the sample is exposed to daylight. It is possible that also parts of the added trans-resveratrol are converted to the cis-isomer and may even oligomerize to the viniferins. According to respective areas below the peaks of one spiked and one original sample from the same selection, the quantities of all the components are increased after being spiked; for example GV11 and GV13, both leaves collected in September, but GV13 spiked with 5 ml trans-resveratrol stock solution.

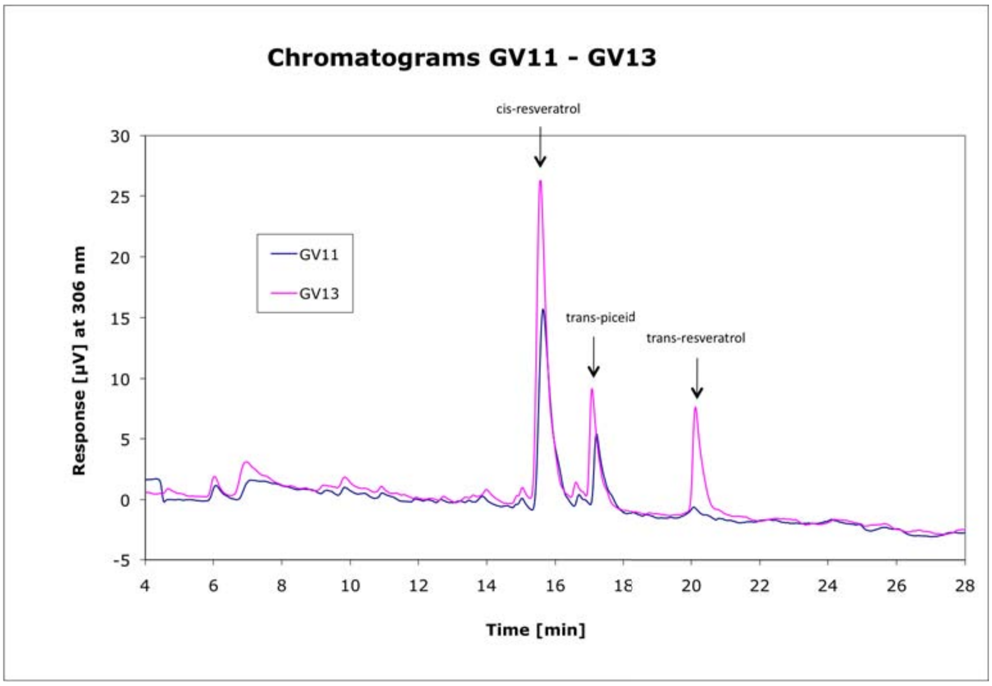


Figure 38: Comparison chromatograms GV11 - GV13

It is very probable that a chemical equilibrium between the different isomers is aspired. The heights of the peaks of the spiked sample show pretty much the same profile qualitatively but all peaks are bigger.

With this method a separation of the isomers of resveratrol and piceid is possible and no further impacts in between these incongruent molecules can be investigated. In this work however, besides the deviation in between different samples, the isomerization to other related molecules had a significant impact on the obtained values.

5 Discussion

5.1 OPC assay

The highest content of proanthocyanidins is found in the seeds of white grapes. The oil has almost no content, which means that for applied purposes, pressing out the seeds leads to a concentration of polyphenolic substances in the presscakes-leftovers, which makes it higher in value.

The oligomers are split up into monomers with the attack of strong acids and they can be measured as well.

Vanillin constructs an orange complex with the proanthocyanidins like catechins, chalcones, tannines but also stilbenes like resveratrol. So it also correlates with the antioxidative capacity of the certain samples. The leaves were yellowish to red after storage in the fridge for more than four months. Fresh samples in earlier stages and even shoots may have higher quantities of available antioxidants, maybe, even in predeveloped biosynthetic phases. The next aim was to find out if, without the effect of pathogenes, resveratrol is produced and functions as a phytoalexine [55]. Moreover, the question was which sectors and which stages of development of the plant *Vitis vinifera* were involved.

5.2 Photometer

The standard solutions show qualitatively similar absorption ranges for the piceid and the trans-resveratrol itself. The intensity is lower for the glycoside, which is also obvious since the same concentration of the two stocks which differ in molar mass leads to a lower amount of the glycosidial heavier molecule. At the tenth of the concentration ratio the absorption range shows that the same tendencies can be estimated.

All the cultivars show qualitatively similar absorption for one type of sample. For smaller wavelengths, lower than 200 nm, the absorption reaches undetectable limits, which is due to methanol being used as the solvent. Lignified parts, like stems or some shoots have very high absorption, lower than 250 nm, which would reduce the scale a lot. The stems collected earlier in the year show the maximum at around 280 nm, which is the responding wavelength of flavanoids. There is absorption in the region of around 306 nm at the maximum absorption for trans-resveratrol.

The undiluted absorption spectra at visible wavelengths show that the extract of the leaves is more intense and richer in absorption maxima. The shoots collected in September were more lignified and the spectra seem to be more similar to the rest of the stems than the fresh and green ones from July, which take after the spectra of the leaves extract. They strongly absorb at around 665 nm, corresponding to chlorophyll A[70]. The absorption at around 530 nm corresponds to the visible wavelength for green. The extract of the stems is not greenish and therefore does not absorb at this wavelength region.

5.3 Evaluation

At any time of the year, the stems show the highest amounts of trans-resveratrol and its oligomers, which are bigger in size and therefore being eluted with this method after the monomers, see Figure 34 on page 45. These are the most potent compounds considering bioactivity.

Trends for higher concentration by grape colour can be observed, namely red grapes show higher quantities of identified compounds.

Suggesting 2 tons of lignified matter as leftover each year per hectare makes an average total of about 1,4 kg pure trans-resveratrol and double that considering the viniferins as well. Calculating with the price of the highest purified and certified as p.a. quality, packed at 100 mg packages the added value of field hectare could be 1,4 million € [72]. Being realistic, the bulk price ranges from 400 to 1900 € per kg [73] which sums up to 560 to 2660 € added value per hectare. Generally, the smaller quantities and better qualities sold, the higher the price.

If an extract is used as an aid in on-farm-sustainibility - which can replace fungicide totally - the agriculturist could spare these expenses and also the contamination that aggressive chemicals, usually based on copper, bring on the farm, even in organic farming.

When using a crude extract of the leftovers, many more active polyphenols are contained and the only way to distinguish the effect is to try it out and compare it to other case studies.

5.4 Stability

The decay of the main component of concern to oxidative products may be of scientific interest, but in practice it will not make a big difference regarding its bioactivity. The viniferins are highly active ingredients in the plant itself and therefore, if an extract is used for any kind of application, its stability is not an element of concern, as long as UV-exposure is kept to a minimum.

However, there could be one problem. Medical applications need standardization of contained compounds, which is hardly possible without chromatographic separation. To overcome this problem, the extract could still be used as a dietary supplement instead of a genuine pharmaceutical product.

The technical use as a fungicide for example is non problematic anyway, since viniferins have roughly the same effects.

5.5 Conclusion

The method worked out quite well, though to some respects the material itself was not representative enough considering the different constitutions of soil, climate, elevation and cultivation methods. Since trans-resveratrol should be produced as an answer to external stress factors and the main influence comes from fungal attacks, the use of fungicides as applied on the sample fields may lead to lower amounts in general.

6 Perspective

The results of this study form new possibilities of considering a more wholistic use of the plant as well, as it opens up many new questions. For further output new investigations should include the comparison between organic and conventional farming. The idea of organic farming is that nature has its mechanisms to get rid of infections by itself and the more the plant is left to help itself, the more phytoalexines will be synthesized, which would also have a positive impact on humans who consume products with higher antioxidative activity. Just in a few sweet wines though, mould is imperative on the grapes for the distinct taste, which leads in the first case to the usage of high quantities of harmful fungicide. The idea could be to produce a durable extract of the stems stored in the dark and applied as an aid in organic farming. There could also be scape vines in one part of the field for higher yields of antifungal components and the rest be kept for normal processing. There is definitely a high potential in optimizing viticulture considering all aspects of a sustainable circular flow management. It is not futuristic aspects but realistic implements that may lead to a more ecological and more economical use of our renewable resources.

References

[1] alchemia-nova and IfaS. Vitis Finessa. EU Frame Programme 7, Biorefinery Joint Call (FP7-2009-BIOREFINERY_CP). September 2008. Personal notice.

[2] Braungart M.; Speech at Cradle to Cradle event from alchemia-nova in “freiRAUM” Weyrgasse 9, 1030 Vienna. December 4th, 2009.

[3] Morlat R.; Symoneaux, Ronan. Long-term additions of organic amendments in a loire valley vineyard on a calcareous sandy soil. III. Effects on fruit composition and chemical and sensory characteristics of cabernet franc wine. American Journal of Enology and Viticulture (2008), 59(4), 375-386

[4] De Prado R.; M. Yuste-Rojas et al. Effect of Soil Type on Wines Produced from Vitis vinifera L. Cv. Grenache in Commercial Vineyards. Journal of Agricultural and Food Chemistry (2007), 55(3), 779-786

[5] Moio L. ; S. Romano et al. Influence of some vine growing and enological factors on the red wine quality produced from Taurasi Aglianico grapes. Vignevini (1999), 26(1/2), 80-84, 87-92

[6] Faitova K., Hejtmankova A., Lachaman J., Pivec V., Dudjak J.The contents of total polyphenolic compounds and trans-resveratrol in white Riesling originated in the Czech Republic. Czech J. Food Sci. (2004), 22: 215–22

[7] Häkkinen S. Flavanols and phenolic acids in berries and berry products. Kuipo University Publications D. Medical Sciences (2000), 221

[8] Eder R., Wendelin S., Vrhosvek U. Resveratrolgehalte von Trauben und Rotweinen in Abhängigkeit von Lesejahrgang und Lesetermin. Mitt. Klosterneuburg (2001) 51: 64-78

[9] Eder R., Wendelin S. Phenolzusammensetzung und antioxidative Kapazität von Trauben und Weinen. Jahrestagung der Arbeitsgemeinschaft landwirtschaftlicher Versuchsanstalten ALVA. Klosterneuburg (2002)

[10] Otreba J., Berghofer E., Wendelin S., Eder R. Polyphenole und antioxidative Kapazität in öst. Weinen aus konventioneller und biologischer Traubenproduktion. Mitteilungen Klosterneuburg (2006), 56: 22-32

[11] Brandl H. Kaltenkirchen,UV-induced synthesis of the phytoalexin resveratrol. Germany. Praxis der Naturwissenschaften, Chemie (1994), 43(3), 35-6

[12] Goldberg, D. M., Ng E., Karumanchiri A., Diamandis E.P., Soleas G.J. Resveratrol glucosides are important components of commercial wines. American Journal of Enology and Viticulture (1996), 47(4), 415-420

[13] Dercks W. and Creasy L. L. The significance of stilbene phytoalexins in the Plasmopara viticola-grapevine interaction. Physiol. Mol. Plant Pathol. (1989), 34, 189-202

[14] Hoos G., Blaich, R.J. Influence of resveratrol on germination of conidia and mycelial growth of Botrytis cinerea and Phomopsis viticola. J. Phytopathol. (1990) 129, 102-110.

[15] Dai G.H., Andary, C., Mondolot-Cosson, L., Boubals, D. Histochemical studies on the interaction between three species of grapevine, Vitis vinifera, V. rupestris and V. rotundifolia and the downy mildew fungus, Plasmopara viticola. Physiol. Mol. Plant Pathol. (1995), 46, 177-188

[16] Pezet R., Gindro K., Viret O., Spring J.-L. Glycosylation and oxidative dimerization of resveratrol are respectively associated to sensitivity and resistance of grapevine cultivars to downy mildew. Physiol. Mol. Plant Pathol. (2005), 65, 297-303

[17] Langcake P.; Cornford, C. A.; Pryce, R. J. Identification of pterostilbene as a phytoalexin from Vitis vinifera leaves. Phytochemistry (1979), 18(6), 1025-7

[18] Aggarwal B. B.; Bhardway, A.; Aggarwal, R. S.; Seeram, N.P.; Shishodia, S.; Takada. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Y. Anticancer Res. 2004, 24, 2783-2840.

[19] Langcake P. Disease resistance of Vitis spp. and the production of the stress metabolites resveratrol, epsilon-viniferin, alpha-viniferin, and pterostilbene. Physiol. Plant Pathol. (1981) 9, 77-86.

[20] Adrian M., Jeandet, P., Veneau, J., Weston, L.A., Bessis, R. Biological activity of resveratrol, a stilbenic compound from grapevines, against Botrytis cinerea, the causal agent for gray mold. J. Chem. Ecol. (1997) 23, 1689-1702.

[21] Hipskind J.D., Paiva, N.L. Constitutive accumulation of a resveratrol- glucoside in transgenic alfalfa increases resistance to Phoma medicaginis. Mol. Plant-Microbe Interact. (2000) 13, 551-562.

[22] Sarig P., Zahavi T., Zutkhi Y., Yannai S., Lisker N., Ben-Arie R. Ozone for control of postharvest fruit decay of table grapes caused by Rhizopus stolonifer. . Physiol. Mol. Plant Pathol. (1997) 50, 337-347.

[23] Urena A.G., Orea JM., Montero C., Jiménez JB, González JL, Sánchez A, Dorado M. Improving postharvest resistance in fruits by external application of trans-resveratrol. J. Agric. Food Chem. (2003) 51, 82-89

[24] Jimenez J.B. Resveratrol treatment controls microbial flora, prolongs shelf life, and preserves nutritional quality of fruit. J. Agric. Food Chem. (2005) 53, 1526-1530.

[25] Langcake P., Pryce, R.J. The production of resveratrol by Vitis vinifera and other members of the Vitaceae as a response to infection or injury. Physiol. Plant Pathol. (1976) 9, 77-86

[26] Langcake P. Disease resistance of Vitis spp. and the production of the stress metabolites resveratrol, epsilon-viniferin, alpha-viniferin, and pterostilbene. Physiol. Plant Pathol. (1981) 18, 213-226.

[27] Rayne S. Grape cane waste as a source of trans-resveratrol and trans-viniferin: High-value phytochemicals with medicinal and anti-phytopathogenic applications. Industrial Crops and Products (2008), 27, 3, 335-340

[28] Mehofer M.. Bundesamt für Wein- und Obstbau Klosterneuburg, Abt. Weinbau. Personal statement. December 2009

[29] Aaviksaar A., et al. Purification of resveratrol from vine stems. Estonian Acad. Sci. Chem. (2003) 52, 155-164.

[30] Püssa T., J. Floren, P. Kuldkepp, and Ain Raal. Survey of Grapevine Vitis vinifera Stem Polyphenols by Liquid Chromatography -Diode Array Detection-Tandem Mass Spectrometry. J.Agric.FoodChem. (2006), 54, 7488-7494

[31] Pour Nikfardjam S.P.M. Polyphenole in Weißweinen und Traubensäften und ihre Veränderung im Verlauf der Herstellung. Diss. Justus-Liebig-Universität, Giessen. Marburg: Tactum (2002)

[32] Romero-Pérez A, Ibern-Gómez M, Lamuela-Raventós RM, de La Torre-Boronat MC. Piceid, the major resveratrol derivative in grape juices. J Agric Food Chem. (1999), 47(4), 1533-6.

[33] Henry-Vitrac C; A.Desmouliere; D.Girard; J.-M. Merillon; S. Krisa. Transport, deglycosylation, and metabolism of trans-piceid by small intestinal epithelial cells. European Journal of Nutrition (2006), 45(7), 376-382.

[34] Xijing Chen, Hui He, Guangji Wang, Bing Yang, Weichao Ren, Le Ma, Qiaoling Yu. Stereospecific determination of cis- and trans-resveratrol in rat plasma by HPLC: application to pharmacokinetic studies. Biomedical Chromatography (2007) 21(3), 257 - 265

[35] Camont L, Cottart CH, Rhayem Y, Nivet-Antoine V, Djelidi R, Collin F, Beaudeux JL, Bonnefont-Rousselot D. Simple spectrophotometric assessment of the trans-/cis-resveratrol ratio in aqueous solutions. Analytica Chimica Acta (2009) 634 121–128

[36] Brent C. Trela and Andrew L. Waterhouse. Resveratrol: Isomeric Molar Absorptivities and Stability. J. Agric. Food Chem. (1996) 44, 1253–1257

[37] RÖMPP Online. [http://www.roempp.com/prod/]. Retrieved at January 27th, 2010.

[38] Kopp, P., 1998. Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the 'French paradox'?. Eur. J. Endocrin. 138, 619-620.

[39] Sigma-Aldrich Online [http://www.sigmaaldrich.com], Retrieved at: January 27th, 2010

[40] Baur, J.A., Sinclair, D.A., 2006. Therapeutical potential of resveratrol : The in vivo evidence. Nature Rev. Drug Discov. 5, 493-506.

[41] Bradamante, S., L. Barenghi, L., Villa, A. Cardiovascular protective effects of resveratrol. Cardiovasc. Drug Rev. (2004) 22, 169-188.

[42] Jang M., Cai, L. et al. Cancer chemopreventative activity of resveratrol, a natural product derived from grapes. Science (1997) 275, 218-220.

[43] Valenzano D., Terzibasi, E., Genade, T., Cattaneo, A., Domenici, L., Cellerino, A. Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. Curr. Biol. (2006) 16, 296-300.

[44] Palomino O., M.P. Gomez-Serranillos, E. Slowing, E. Carretero and A. Villar. Study of polyphenols in grape berries by reversed-phase high-performance liquid chromatography. Journal of Chromatography (2000), A 870, 449–451.

[45] Sanchez-Moreno C. , A. Jimenez-Escrig and F. Saura-Calixto. Study of low-density lipoprotein oxidizability indexes to measure the antioxidant activity of dietary polyphenols. Nutrition Research (2000), 20 (7), 942–953.

[46] Nakagawa H., Y. Kiyozuka, Y. Uemura, H. Senzaki, N. Shikata, K. Hioki and A. Tsubura, Resveratrol inhibits human breast cancer cell growth and may mitigate the effect of linoleic acid, a potent breast cancer cell stimulator, Journal of Cancer Research and Clinical Oncology (2001), 127, 258–264.

[47] Khanna S., S. Roy, D. Bagchi, M. Bagchi and C.K. Ken. Upregulation of oxidant-induced VEGF expression in cultured keratinocytes by a grape seed proanthocyanidin extract. Free Radical Biology and Medicine (2001), 31 (1), 38–42.

[48] Oshima Y., Namao, K., Kamijou, A., Matsuoka, S., Nakano, M., Terao, K., Ohizumi, Y. Powerful hepatoprotective and hepatotoxic plant oligostilbenes, isolated from the Oriental medicinal plant Vitis coignetiae (Vitaceae). Experientia (1995), 51, 63- 66.

[49] Privat C., Telo, J.P., Bernardes-Genisson, V., Vieira, A., Souchard, J.P., Nepveu, F. Antioxidant properties of trans- -viniferin as compared to stilbene ε derivatives in aqueous and nonaqueous media. J. Agric. Food Chem. (2002) 50, 1213- 1217.

[50] Billard C., Izard, J.C., Roman, V., Kern, C., Mathiot, C., Mentz, F., Kolb, J.P., 2002. Comparative antiproliferative and apoptotic effects of resveratrol, ε-viniferin and vine-shots derived polyphenols (vineatrols) on chronic B lymphocytic leukemia cells and normal human lymphocytes. Leuk. Lymphoma (2002) 43, 1991-2002.

[51] Piver B., Berthou, F., Dreano, Y., Lucas, D. Differential inhibition of human cytochrome P450 enzymes by ε-viniferin, the dimer of resveratrol: Comparison with resveratrol and polyphenols from alcoholized beverages. Life Sci. (2003) 73, 1199-1213

[52] Yanez M., Fraiz, N., Cano, E., Orallo, F. Trans-ε-viniferin, a polyphenol present in wines, is an inhibitor of noradrenaline and 5-hydroxytryptamine uptake and of monoamine oxidase activity. Eur. J. Pharmacol. (2006) 542, 54-60.

[53] Köck A. Eubio as Austrian distributor from Axxora chemicals. [http://www.eubio.at/]. Retrieved at: March 17th, 2009.

[54] Mattivi, F.; Reniero, F. Oligostilbenes from the roots of genus Vitis. Bulletin de Liaison - Groupe Polyphenols (1992), 16(1), 116-19.

[55] Stich K. “Biochemie der Pflanzen“ . TU Vienna. Lecture winter semester 2007.

[56] Langcake, P.; McCarthy, W. V The relationship of resveratrol production to infection of grapevine leaves by Botrytis cinerea.. Shell Biosci. Lab. Shell Res. Ltd., Sittingbourne/Kent, UK. Vitis (1979), 18(3), 244-53

[57] Process of Obtainment of Trans-Resveratrol and/or Emodin and nutraceuticals compositions containing them, Patent No. WO 2008092221 (A2). August 7, 2008.

[58] Method for producing a drug extract that contains hydroxystilbene, Patent No. WO 2006082071 (A1). August 10, 2006.

[59] Pezet R., C. Perret, J. B. Jean-Denis, R. Tabacchi, K. Gindro, O.Viret. δ-Viniferin, a Resveraictrol Dehydrodimer: One of the Major Stilbenes Synthesized by Stressed Grapevine Leaves. J. Agric. Food Chem. 2003, 51, 5488-5492

[60] Pezet R.; Pont, V.; Cuenat, P Method to determine resveratrol and pterostilbene in grape berries and wines using high-performance liquid chromatography and highly sensitive fluorimetric detection.. Journal of Chromatography, A (1994), 663(2), 191-7

[61] Waterhouse A. L., Lamuela-Raventos, R. The occurrence of piceid, a stilbene glucoside, in grape berries.. Dep. Viticulture Enology, Univ. California, Davis, CA, USA. Phytochemistry (1994), 37(2), 571-3

[62] Hart J.H. , Role of Phytostilbenes in Decay and Disease Resistance, Annual Review of Phytopathology, (1981), 19, 437-458

[63] Siemann E. H.; Creasy, L. L. Concentration of the phytoalexin resveratrol in wine. American Journal of Enology and Viticulture (1992), 43(1), 49-52

[64] Pont, V.; Pezet, R.. Relation between the chemical structure and the biological activity of hydroxystilbenes against Botrytis cinerea. Journal of Phytopathology (1990), 130(1), 1-5

[65] Baoshan Sun J. M. Ricardo-da-Silva and I. Spranger. Critical Factors of Vanillin Assay for Catechins and Proanthocyanidins. J. Agric. Food Chem., 1998, 46 (10), 4267-4274

[66] Butler L. G., M. L. Price, J. E. Brothertod. Vanillin Assay for Proanthocyanidins (Condensed Tannins): Modification of the Solvent for Estimation of the Degree of Polymerization. J. Agric. Food Chem. 1982, 30, 1087-1089

[67] Schüller K. Diploma thesis: Phytonutriments and Chemoprevention: the Role of Polyphenols in the Prevention of Colorectal Cancer. University of Vienna, Department of Pharmacognosy, supervisor Prof. Dr. Brigitte Kopp

[68] Broadhurst R. B., William T. Jones. Analysis of Condensed Tannins Using Acidified Vanillin. J. Sci. Fd Agric. 1978, 29, 788-794

[69] Meyer V. R. Praxis der Hochleistungs-Flüssigchromatographie. Wiley-VCH. 10th edition. 2009

[70] Cerovic Z.G.,Ounis A., Cartelat A., Latouche G., Goulas Y., Meyer S., Moya I. The use of chlorophyll fluorescence excitation spectra for the non-destructive in situ assessment of UV-absorbing compounds in leaves. Plant, Cell and Environment (2002) 25, 1663–1676

[71] A. I. Romero-Pérez, Rosa M. Lamuela-Raventós, A. L. Waterhouse and M. C. de la Torre-Boronat. Levels of cis- and trans-Resveratrol and Their Glucosides in White and Rose´ VitisviniferaWines from Spain. J.Agric.FoodChem. 1996, 44, 2124−2128

[72] Sigma-Aldrich Austria [http://www.sigmaaldrich.com]. Retrieved at: June 11th 2010

[73] [http://www.megaresveratrol.net, http://www.nextag.com/resveratrol/stores-html, http://www.myshopping.com.au, http://www.super-smart.eu]. Retrieved at: June 11th 2010