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DISSERTATION

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Activity profiling of Austrian red wine targeting cardiovascular diseases: A contribution to understand the “French Paradox”

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1 INTRODUCTION

1.1 Contribution of endothelial dysfunction to CVDs (cardiovascular diseases)

1.1.1 Cardiovascular diseases

Cardiovascular diseases (CVDs) are the number one cause of death worldwide and therefore, more people die annually from CVDs than from any other reason in western industrialized countries[1]. Concerning Europe especially countries located in the North, e.g. Ireland and Finland, are affected. Cardiovascular disease is a term describing coronary heart disease, stroke and peripheral vascular disease, which are all pathological disorders depending on the development of atherosclerosis [1]. Figure 1 shows the distribution of main causes of death in Austria in the year 2012 for both, female and male. As in the previous decades, CVDs lead this statistics. In addition, this diagram is representative for the ratio of mortality causes in typical western societies.

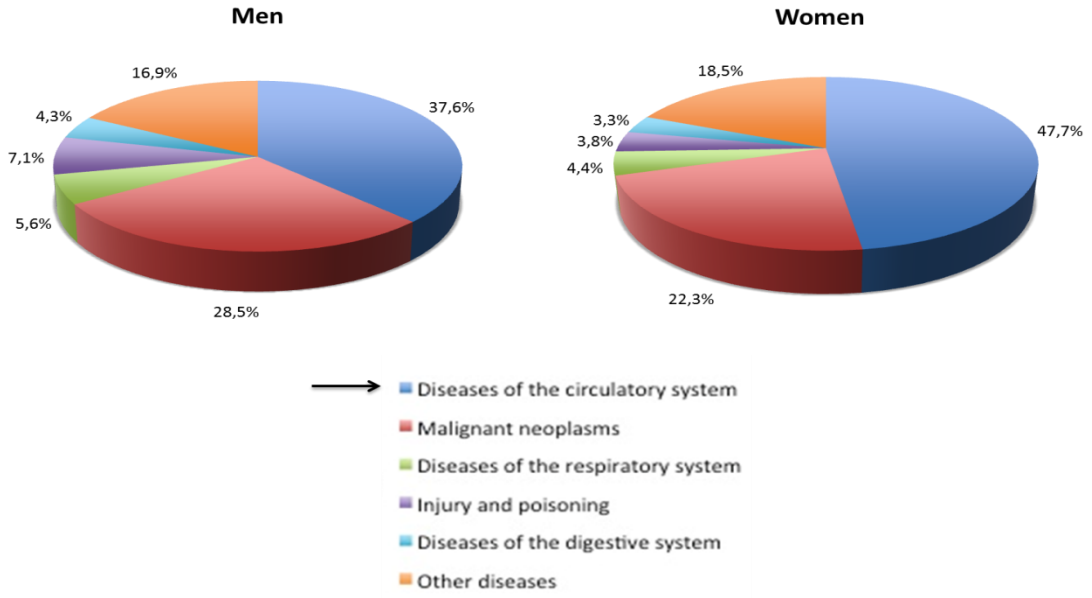


Figure 1: Overall mortality in Austria (2012), structured by leading causes of death and related to the sex (adapted from Statistik Austria ("Todesursachenstatistik"; 18.02.2015)[2]).

[1] http://www.who.int/mediacentre/factsheets/fs317/en/index.html access: 2011/08/07
[2] http://www.statistik.at/web_de/statistiken/gesundheit/todesursachen/todesursachen_ausgewaehlte/ access: 2015/18/02

Over the past 60 years, several epidemiological studies have revealed important environmental risk factors associated with atherosclerosis [2]. These are on the one hand factors with a strong genetic background like an elevated level of LDL combined with a lower level of HDL, increased blood pressure, enhanced level of homocysteine, diabetes and obesity, systemic inflammation, the gender, and family history. On the other hand, there are environmental factors like a high-fat diet, smoking, lack of physiological exercise, low antioxidant levels and infections, e.g. with *Chlamydia pneumoniae* [3-11].

The endothelium (physiological monolayer of endothelial cells) is coating the inner wall of blood vessels and is thus the interface between blood lumen and vascular smooth muscle cells (VSMC). Endothelial cells (EC) operate as a selectively permeable barrier and are recognized as essential regulators of vascular function. They are sensitive for the vascular flow and dynamic events, such as fluid shear stress, and are therefore inevitable to regulate the maintenance of blood pressure. The latter is provided by vasoactive NO, endothelin-1 and prostacyclin as a response of the endothelium to signaling molecules in the blood. An intact endothelium supports the immune system acquiring leukocytes to inflammations or infections as well as preventing the formation of lesions. [1, 12].

Studies in animal models of atherosclerosis have confirmed, that an impairment of the endothelial function, also termed as endothelial dysfunction, may counteract the functionality of EC and consequently causes a pro-inflammatory and pro-thrombotic state [13]. The abovementioned multifactorial mechanisms establish atherosclerosis, which is generally associated to endothelial dysfunction. More precisely, a diminished level of the released endothelial factor nitric oxide (NO), which is secreted by the endothelial nitric oxide synthase (eNOS), or a decrease in enzyme activity or attenuation of the eNOS expression support atherogenesis [14, 15].

1.2 The endothelial nitric oxide synthase (eNOS)

1.2.1 Characteristics of the eNOS enzyme

In the 1980ies, it has been described for the first time by Zawadzki and Furchgott, that the endothelium has a crucial influence on the regulation of the vascular tone [9]. The unknown responsible factor, termed endothelium-derived relaxing factor (EDRF), was later on identified as nitric oxide (NO) [15,16].

Since then, the functions and properties of NO have been intensively investigated. It is produced by the nitric oxide synthase (NOS), of which three isoforms have been identified up to now. These are the endothelial nitric oxide synthase (eNOS), the inducible nitric oxide synthase (iNOS) and the neuronal nitric oxide synthase (nNOS). Although all of them are responsible for the production and release of NO, they are encoded by different genes and have diverse characteristics, such as localization in the cell, and catalytic properties [16].

In the vascular system, the eNOS, which has a molecular weight of 133 kDa, is the most relevant isoform. It is mainly located in endothelial cells, where it is activated by acetylcholine, bradykinin, vascular endothelial growth factor (VEGF) and the blood flow, and serves as a key regulator of the vascular tone. The subcellular location for eNOS is the caveolae, which are small emarginations of the cell membrane showing enrichment of sphingomyelin and cholesterol [17]. The eNOS activity is downregulated by interaction with caveolin-1 (Cav-1), the major protein bound to caveolae [18]. Consequently, Cav-1 knockout mice show higher NO levels in blood and increased endothelium-dependent relaxation [19]. Binding to calmodulin (CaM), on the other hand, enhances eNOS activity.

The generated NO is a very reactive lipophilic molecule. It occurs in a gaseous state and is thus able to diffuse into contiguous tissues outside of the catalysing cell compartment. Nitric oxide possesses the properties of a radical and therefore has a short half-life of five seconds [20]. NO is biosynthesized by conversion of L-arginine to L-citrulline by eNOS applying the cofactors flavin adenine mononucleotide (FAM),

flavin adeninedinucleotide (FAD), (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4) and nicotinamide adenine dinucleotide phosphate (NADPH) [21]. For the functionality of the eNOS, which consists of two domains (an oxygenase domain and a reductase domain), a homodimer has to be formed. The two domains are linked via CaM, and the homodimer is complexed by a zinc ion for stabilization [22].

Endothelial NO is constitutively released and plays a key role in the vascular system maintaining the vascular tone due to its anti-atherogenic, respectively vasoprotective nature in the endothelium. From the endothelium, NO is diffusing in both directions, the blood lumen and the abluminal vascular smooth muscle cells (VSMC). In the VSMC it is promptly affecting a relaxation by activating the soluble guanylate cyclase (sGC). Moreover NO inhibits the pathological proliferation of VSMC by suppressing the platelet-derived growth factor B (PDGF-B), which causes narrowing of blood vessels as a preliminary stage of atherosclerosis [23]. Endothelial NO prevents the expression of NF-κB as well as related adhesion molecules like the vascular cell adhesion molecule 1 (VCAM-1) or the intercellular cell adhesion molecule 1 (ICAM-1). VCAM-1 and ICAM-1 are mediating the adhesion of macrophages onto the endothelial cell layer, an initial step of inflammation as a preliminary stage of atherogenesis. Additionally, the luminal secreted endothelial NO counteracts the oxidation of low-density lipoprotein (LDL). Hence, the subsequent uptake of oxLDL into the intima, the formation of foam cells continued by fatty streaks and a final stenosis of blood vessel due to the migration of VSMC through the endothelium do not occur. NO also inhibits the endothelial expression of the tissue factor (TF), which is an initiator of platelet aggregation. It is induced by pro-inflammatory tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) or IL-8. Due to its important regulatory functions, any impairment of the availability of endothelial NO provokes the dysfunction of the vascular system and contributes to the development of atherosclerosis and further cardiovascular diseases [16, 24].

1.2.2 Characteristics of the isoforms iNOS and nNOS

The two other isoform of the NOS are the above mentioned iNOS and nNOS:
The iNOS (inducible nitric oxide synthase) has a molecular weight of 131 kDa and is expressed in a large number of tissues. NO is predominantly synthesized upon induction in the context of inflammation. It represents an important defense factor of the immune system against pathogens like bacteria. In contrast to the constitutively expressed eNOS, the inducible form of NOS reaches up to twenty fold higher concentrations of NO due to abscence of the autoregulating Ca^{2+}/calmodulin feedback mechanism [16, 24].

The nNOS is primarily expressed in neuronal tissue and represents a 161 kDa protein. It is comparable to eNOS as NO is constitutively released at a basic level, but in neuronal tissue. nNOS has no anchor sites for the cell membrane, but gets gathered to the nerve synapses by its unique PDZ domain. It is a Ca^{2+}/calmodulin-dependent enzyme and gets delivered via the voltage-dependent opening of calcium-channels. nNOS released NO is suggested to influence behavior, learning and memory [24, 25].

1.2.3 The formation of endothelial NO

eNOS catalyzes the oxidation of the substrate L-arginine to L-citrulline and NO in the presence of several cofactors. It is a homodimeric enzyme, which consists of two monomers each containing an oxygenase domain and a reductase domain as subunits. The electron donor NADPH offers electrons to the carboxy-terminal reductase domain, from which they are transferred by FAD and FMN with CaM as interface to the amino-terminal oxygenase domain. The electrons are accepted by the heme molecule, an iron containing prosthetic cofactor. CaM is able to boost this electron transfer from the flavins to the heme. Molecular oxygen, which is complexed by the heme, gets reduced via the transferred electrons and oxidizes the guanidine group of the L-arginine. The product N-hydroxy-L-arginine reacts with another oxygen molecule at the hydroxy group of the amino-carbamoyl residue to form L-citrulline together with NO [26-28].

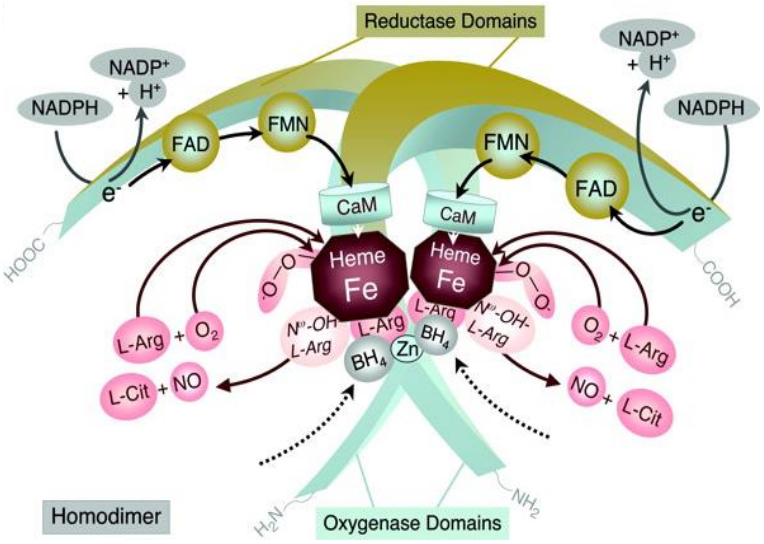


Figure 2: This illustration was taken from "Endothelial nitric oxide synthase in vascular disease: from marvel to menace" by U. Förstermann and T. Münzel. It was published in "Circulation" in 2006 [29]. It describes the basic structure of the eNOS enzyme and the formation of NO.

1.2.4 Regulation of eNOS activity

Activity of the eNOS isoform is the most influenced one by the intracellular Ca^{2+} level (Ca_i^{2+}). Ca_i^{2+} is binding to Calmodulin (CaM) and the formed Ca^{2+}/CaM complex is responsible for the initial step of the eNOS enzyme activation. The Ca^{2+}/CaM complex intercepts the interaction between eNOS and Cav-1, which is located at the caveolae in the cell membrane. Ca^{2+}/CaM itself binds to eNOS, disables the inhibiting effect of Cav-1, and enables the formation of the eNOS homodimer. The bound CaM initiates the electron transfer from the oxygenase to the reductase domain, which is necessary for the synthesis of NO, respectively eNOS activity [30, 31]. The binding of CaM provides a fast and predominant mechanism of the eNOS enzyme activation and is triggered by acetylcholine, bradykinin, estradiol, histamine as well as by calcium ionophore A23187 (see chapter 2.5).

Also substantial for the activation of the eNOS enzyme is the chaperone heat shock protein 90 (Hsp90). This highly expressed protein assists the folding of the eNOS enzyme structure. Hsp90 is bound to the functional eNOS protein, and enhances the eNOS affinity for CaM [32]. Stimuli for eNOS activation additionally increase the

interaction between eNOS and Hsp90 [33]. Moreover, Hsp90 supplies a scaffold protein for eNOS and protein kinase B (Akt), which are bound contemporaneously, after Ser^{1177} phosphorylation of eNOS.

Since six phosphorylation sites of the eNOS are identified up to now, Ser^{1177} is the most prominent phosphorylation site. The eNOS phosphorylation is known to be involved as a main regulating posttranslational mechanism in eNOS activation and is coupled with several kinases (PKA, Akt, AMPK, CaMKII and PKG). Ser^{1177} occurs unphosphorylated, and stimuli as VEGF, insulin, shear stress etc. are able to induce phosphorylation, and furthermore eNOS activity [34, 35]. Other known phosphorylation sites of the eNOS protein are Thr^{495}, Ser^{615}, Ser^{633}, Ser^{116} and Tyr^{83}.

1.2.5 Regulation of eNOS expression

eNOS was formerly believed to be solely expressed constitutively, but meanwhile physiological and pathophysiological regulators of the enzyme are described. An upregulation of eNOS protein, which has a half-life of about 20 hours [36], by shear stress as a consequence of increased blood pressure as well as the influence of growth factors like PDGF, VEGF and TGF-β1 on the enhancement of eNOS expression are known. Even hypoxia, reactive oxygen species (ROS) and oxidized LDL induce an increased eNOS expression. Furthermore, hormones as estrogen, endothelin-1, angiotensin II or insulin augment the enzyme expression, in contrast to thrombin showing an eNOS downregulation.

The expression of the eNOS protein is initially controlled by transcription factors binding to the promoter of the eNOS gene (i.e. Sp1, Sp3, GATA, NF-κB and others). The stability of the eNOS mRNA indicates influence on the eNOS enzyme expression level. The half-life of eNOS mRNA ranges from 10 to 35 hours [37] and is affected negatively by oxidized LDL, TNF-α, thrombin or bacterial lipopolysaccharides. A prolonged stability of mRNA is associated with VEGF, estrogen, H_2O_2 and cell proliferation [38].

1.2.6 Uncoupling of the eNOS

Risk factors for atherosclerosis are accompanied by endothelial dysfunction. This pathological condition is the result of an increased production of ROS (reactive oxygen species) in the vasculature. NADPH oxidases (e.g. Nox2 and Nox4) are the main source of ROS, and their enhanced expression promotes oxidative stress in the atherosclerotic vascular walls [39]. The NADPH oxidase-induced formation of superoxide ($O_2^{\bullet -}$) is directly reacting with NOS-derived NO to peroxynitrite ($ONOO^-$), which oxidizes the essential eNOS cofactor BH4 (tetrahydrobiopterin) to $BH3^{\bullet}$ and subsequently to BH2 (dihydrobiopterin). This deficiency of BH4 results in eNOS-mediated generation of superoxide instead of NO, termed as eNOS uncoupling [40]. It has severe consequences for the functionality of the endothelium. eNOS uncoupling is responsible for the reduced availability of NO, and additionally, the increased formation of $O_2^{\bullet -}$ initiates the enhanced expression of eNOS protein related to a persistent decrease of BH4. By this means, eNOS attempts to compensate the lack of eNOS-derived NO, but enhances mainly the production of peroxynitrite. These findings are supported by several *in vitro* and *in vivo* studies referring to the connection between eNOS uncoupling and endothelial dysfunction [41]. The oxidation of BH4 may be reversed using ascorbic acid for chemical stabilization of BH4 [42]. Endothelial production of NO can be increased higher than to normal level consequently to an enhanced availability of BH4. In such way, ascorbic acid was used as stimulus for the positive control of the mainly performed [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay in this thesis (see chapters 2.5 and 3.1).

Trans-resveratrol is the most prominent polyphenolic compound present in red wine, and has already shown prevention against several mechanisms involved in the development of cardiovascular diseases [43]. Some data demonstrate that *trans*-resveratrol is able to reverse eNOS uncoupling. The protective effect can be explained by an upregulation of the endogenous cellular antioxidant system including superoxide dismutases (SOD), glutathione peroxidase [44], and a downregulation of the NADPH

oxidases. Thus, *trans*-resveratrol improves the endothelial level of eNOS cofactor BH4, which is crucial counteracting endothelial dysfunction by eNOS uncoupling.

1.3 Red wine

For 20 years the number of publications using "red wine" as search criterion continuously was increasing as depicted in Figure 3[3].

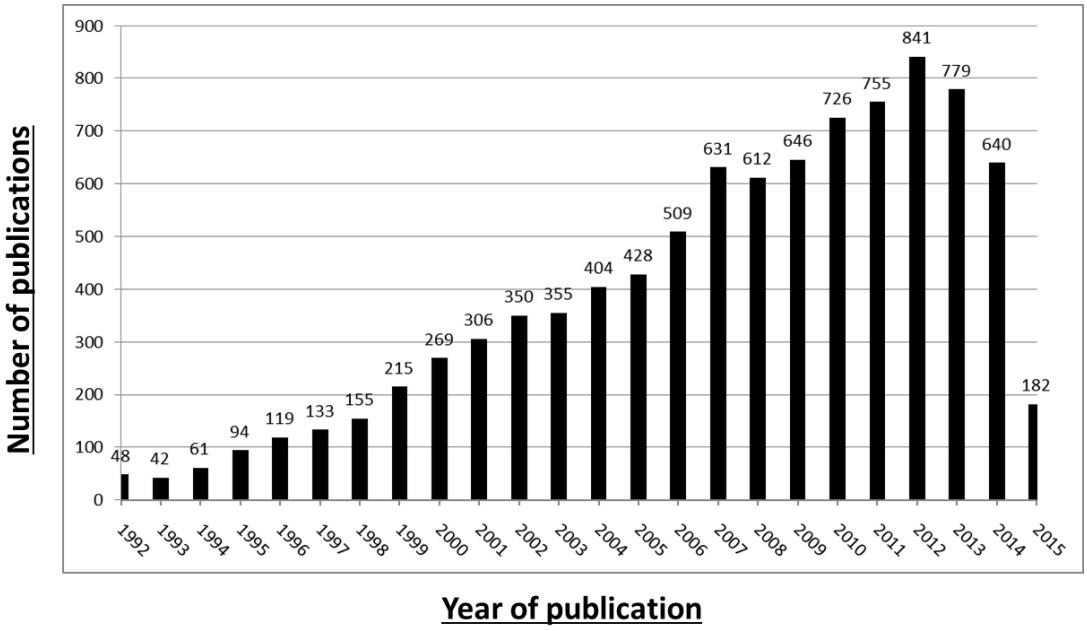


Figure 3: The SciFinder Scholar™ 2015 database was searched using the key word "red wine" (access: 2015/03/13). The plot demonstrates the cumulative number of publications as a function of each year (1992 – 2015).

The epidemiological background was presented two decades ago by Renaud and De Lorgeril describing the correlation of red wine consumption with a lower mortality by coronary heart diseases (CHD) [45].

The findings constituted the term "French paradox", characterizing the discrepancy between intake of saturated fat in France, which is comparable to other european countries, and the significantly, outstanding low mortality because of CHD (see Figure 4A). An additional correlation with the red wine consumption of the same countries serves as possible explanation for the French paradox. The inverted relationship

[3] exploring with SciFinder Scholar™ 2015; references containing "red wine" access: 2015/03/13

between the red wine consumption and the CHD mortality illustrates that the French population, drinking more red wine than people in Northern Europe, has a significantly lower prevalence for CHD mortality, as illustrated in Figure 4B.

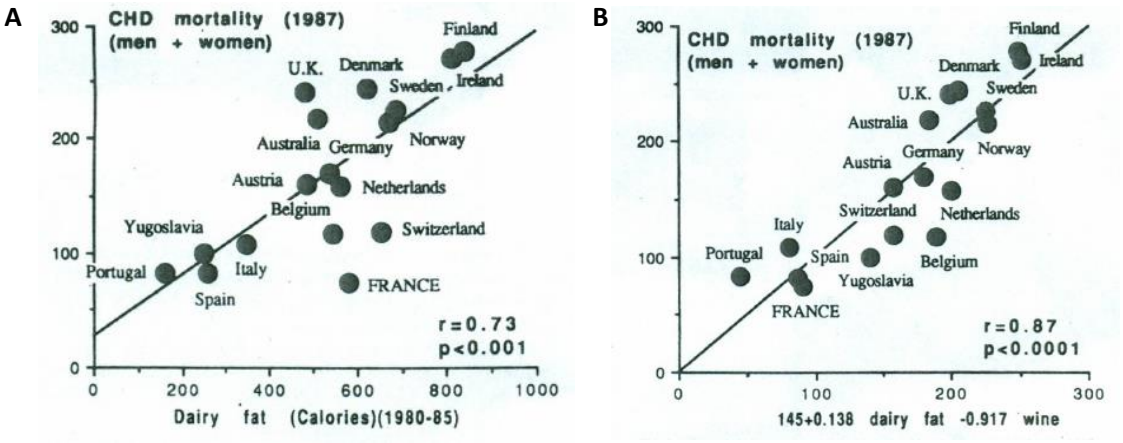


Figure 4: The intake of dairy fat of the French is comparable to other populations in Europe; there is a significantly lower risk of mortality related to CHD (A). Red wine consumption seems to be responsible for this effect (possibly explaining the "French paradox")(B). This figure was taken from "Wine, Alcohol, Platelets, and the French Paradox for Coronary Heart-Disease" by S. Renaud and M. de Lorgeril. It was published in "The Lancet" in 1992 [45].

Following publications discussed the influence of ethanol in lowering the risk for CHD, and more than a moderate consumption of alcohol may be negatively related to CHD. Red wine demonstrated to decreasing the mortality related to CHD more than other liquors like beer and spirit, and hence the cardioprotective effect could be primarily explained by red wine constituents [46-49].

Wine is a predominantly rich source of polyphenolic components, such as flavonoids, anthocyanins, flavan-3-ols, oligomeric proanthocyanidins (OPCs), stilbenes and phenolcarbonic acids, and therefore represents the prime example for a complex mixture of polyphenols. Nowadays, most of these compounds are well known (e.g. *trans*-resveratrol, quercetin, catechin, etc.); not only for their contribution to the complexity of taste but also for their contributing benefits concerning the vascular system.

In healthy humans, the intake of red wine acutely improves an endothelium-dependent vasodilatation [50, 51]. Moreover, a quarter-liter of red wine per day

effectively antagonized a high-fat diet-induced endothelial dysfunction in healthy individuals [52] and enhances the endothelial production of NO [53]. Several studies support a stronger effect when removing the alcohol from wine [54, 55] as well as dealcoholized red wine was discovered to ameliorate the dilatation of blood vessels in coronary artery disease patients in contrast to normal red wine [56]. Furthermore, dealcoholized red wine increases the endothelium-dependent vasodilatation in hypercholesterolaemic rabbits [57] and rats [58].

There exist a large number of both *in vitro* and *in vivo* experiments to underline the relevance of red wine polyphenols as modulators of endothelial NO production. The modest intake of alcohol shows the ability to reduce mortality and cardiovascular risk, but without influence on eNOS activation. The beneficial cardiovascular effects are related to both the normal and the dealcoholized red wine but hardly to white wine assessing the red wine polyphenols (RWP) crucial for the effects [59, 60].

RWP have demonstrated in several studies to increase the endothelial function in different hypertension models using animals [61-65]. Comparable results with RWP were obtained in cultured endothelial cells performing studies concerning acute actions as well as long-term effects.

The rapid activation of endothelial mechanisms (i.e. eNOS activation) depends on the increased calcium level in endothelial cells, which promotes on the one hand NO output of the enzyme in consequence of enhancing the eNOS´ affinity to its activator CaM [66, 67]. On the other hand, the RWP-induced calcium influx improves the phosphorylation of eNOS at Ser^{1177} via the phosphatidylinositol-3-kinase (PI_3K)/Akt pathway leading to an acute NO release [68].

The long-term effect on eNOS, which is already described after treatment with red wine and RWP [69-71], is associated to an upregulated expression of the eNOS gene resulting in a higher amount of eNOS protein and more endothelial NO.

The upregulation of the eNOS protein is considered a result of a synergistic effect of the complex constitution of polyphenols in red wine [72].

The most prominent component in red wine, known to be present in grapes, peanuts and P*olygonum cuspidatum* (Japanese knotweed), is the stilbene derivative (*trans*-) resveratrol. Concerning (red) grapes, it is located in the skin, and its function as phytoalexin is to inhibit microbial infection (e.g. *Botrytis cinerea*) and damage by UV irradiation. In return, these elicitors are necessary for the grapes to induce its biosynthesis.

Since crucial findings of the benefits of red wine were published in the early nineties [45], especially certain components, such as *trans*-resveratrol, attracted a permanently increasing interest. Consequently to the very large number of publications dealing with various effects in laboratory models this most prominent red wine constituent is intensively discussed.

Baur et al. received remarkable attention for their publication that *trans*-resveratrol showed the ability to prolong the lifespan of yeast and even vertebrates like nothobranchius furzeri [73]. It is associated with anti-aging and counteracts mechanisms of metabolic diseases, such as diabetes [74-78]. In contrast, a study revealed that resveratrol does not prolong lifespan of not obese rats, but preserve vascular function (regarding better aerobic performance and exercise capacity) [79]. As only a few human studies have been published to discover physiological benefits, which have been observed in laboratory models, a review aimed to focus on the effects of resveratrol in humans to utilize information for the implementation of human clinical trials [80]. In post-infarction patients (suffering from CAD) a 3-months treatment with 10 mg resveratrol/day improved the diastolic function of the left ventricle, lowered the LDL-cholesterine level, and enhanced endothelial function (measured by flow-mediated vasodilation) [81]. A recent result of a prospective cohort study was performed taking a population-based sample. The level of resveratrol achieved with diet (determined as resveratrol urinary metabolites) did not have impact on health and mortality risk of a population-based sample. Moreover, the concentration of metabolites could not be associated to cardiovascular disease, cancer or inflammatory markers like serum C-reactive protein, IL-6, and tumor necrosis factor

[82]. Meanwhile, as a consequence of the poor bioavailability of *trans*-resveratrol in human [83], investigations of its metabolites have received much more attention [84].
Several studies indicated an improved vascular function upon treatment with *trans*-resveratrol, like a decreased production of superoxide, downregulation of NADPH oxidase, enhanced expression of eNOS enzyme [39, 58, 69, 71, 72, 85] as well as increased eNOS activity after long-term treatment [86]. Mechanistical studies have demonstrated that *trans*-resveratrol effects the relaxation of isolated aortic rings, which is accompanied by acute NO release [87]. This compound induces the phosphorylation of eNOS via AMPK (adenosine monophosphate-acivated protein kinase) [88] or ERK1/2 (extracellular-signal-regulated kinases 1 and 2) [89]. Additionally, this stilbene effects the deacetylation of eNOS, mediated by SIRT1 ((silent mating type information regulation 2 homologue) 1) improving the affinity to CaM [90, 91].
Based on the most cited publication, which presented resveratrol to inhibit tumorgenesis in a mouse skin cancer model in 1997, the number of papers dealing with its chemopreventive activity increased as well [92]. Resveratrol protects against UV radiation-mediated oxidative stress and cutaneous damages including skin cancer [93]. The oral administration of *trans*-resveratrol was proposed to contribute to the prevention of colon carcinogenesis [94], which was supported by an *in vitro* study investigating the anticarcinogenic properties of its metabolites [95].
Altogether, *trans*-resveratrol is considered the polyphenolic drug to interfere development of cardiovascular disease besides further indications, such as cancer.

1.4 The contribution of *Chlamydia pneumoniae* to CVDs

1.4.1 The developmental cycle of *Chlamydia pneumoniae*

Chlamydia pneumoniae (since a reclassification in 1999 the genus is correctly termed as *Chlamydophila*, which is, however, not frequently used) is an intracellular Gram-negative bacterium and causes infections of the respiratory tract in humans, varying from mild pharyngeal symptoms to severe pneumonia. It has a unique life-

cycle existing as “elementary body” (EB) as well as “reticulate body” (RB). The EB is the infectious, extracellular, non-replicating form [96]. EBs can infect various cell types, including macrophages, and thus disseminate to multiple tissues [97, 98]. The RB is the non-infectious, replicating second phenotype of *C. pneumoniae,* formed from the EB after internalization. The intracellular RB is located in a phagosome termed inclusion, which is not able any more to fuse in a lysosome [99]. The RBs have furthermore the ability to form persistent “aberrant bodies” (ABs) in the host cell, if growth conditions get unfavorable ,e.g., in presence of antibiotics such as penicillin [100]. Inside the inclusions, *C. pneumoniae* can maintain the state of a chronic and latent infection, which seems to be untreatable using common antibiotics [101].

Epidemiological studies have indicated an age- and sex-dependent seroprevalence of *C. pneumoniae* antibodies [96, 102]. The rate increases from 50 % in individuals at 20 years of age to 80 % at 80 years of age [103].

1.4.2 *Chlamydia pneumoniae* and atherosclerosis

Based on a seroepidemiologic study in 1988 [97], the hypothesis of a relation between a chlamydial infection and atherosclerosis arose, because patients with CVD showed a significant enhancement of *C. pneumoniae* antibodies compared to a control group. This work represents the fundament of a series of more than fifty clinical studies analyzing *C. pneumoniae* infection in atherosclerosis up to now, which improved the understanding of *C. pneumoniae* pathogenesis concerning atherogenesis [104]. For instance, in a prospective study with children from seven to eleven years of age, seropositive individuals to *C. pneumoniae* demonstrated a significant increase of intima-media thickening compared to seronegative children, and thus supported the influence of a persistent chlamydial infection on atherosclerosis [105].

Today, the three main pathomechanisms to provoke atherosclerosis are known to be the proliferation of vascular cells, the inflammation of the vascular wall and the endothelial dysfunction [106].

An infection with *C. pneumoniae* is generally followed by an inflammation of the respiratory tract. The pathogen enters mononuclear cells as a matter of dissemination in the circulating blood and then gains access to the vasculature [107]. Consequently, endothelial cells become infected, which goes along with enhanced secretion of pro-inflammatory cytokines (e.g. TNF-α, IFN-γ, IL-6) and upregulated expression of leukocyte adhesion molecules (VCAM-1, ICAM-1, E-selectin) [108]. Then, leukocytes migrate through the endothelial cell layer and induce formation of a lesion as illustrated in Figure 5 [109]. An inflammation within the vessel wall causes the enhanced formation of oxidized low-density lipoprotein (oxLDL), which bypasses the LDL receptor and migrates into the intima, where it is taken up by macrophages. Finally, these processes result in the development of foam cells, which are a hallmark of atherosclerosis [110]. Moreover, the progress of atherosclerosis can be increased via the physiological interaction of endothelial cells (EC) with vascular smooth muscle cells (VSMC). A proliferative overexpression of PDGF-B (platelet-derived growth factor), which is a mitogenic stimulus for VSMC to remodel vessels, may induce a pathological thickening of the arterial intima [111].

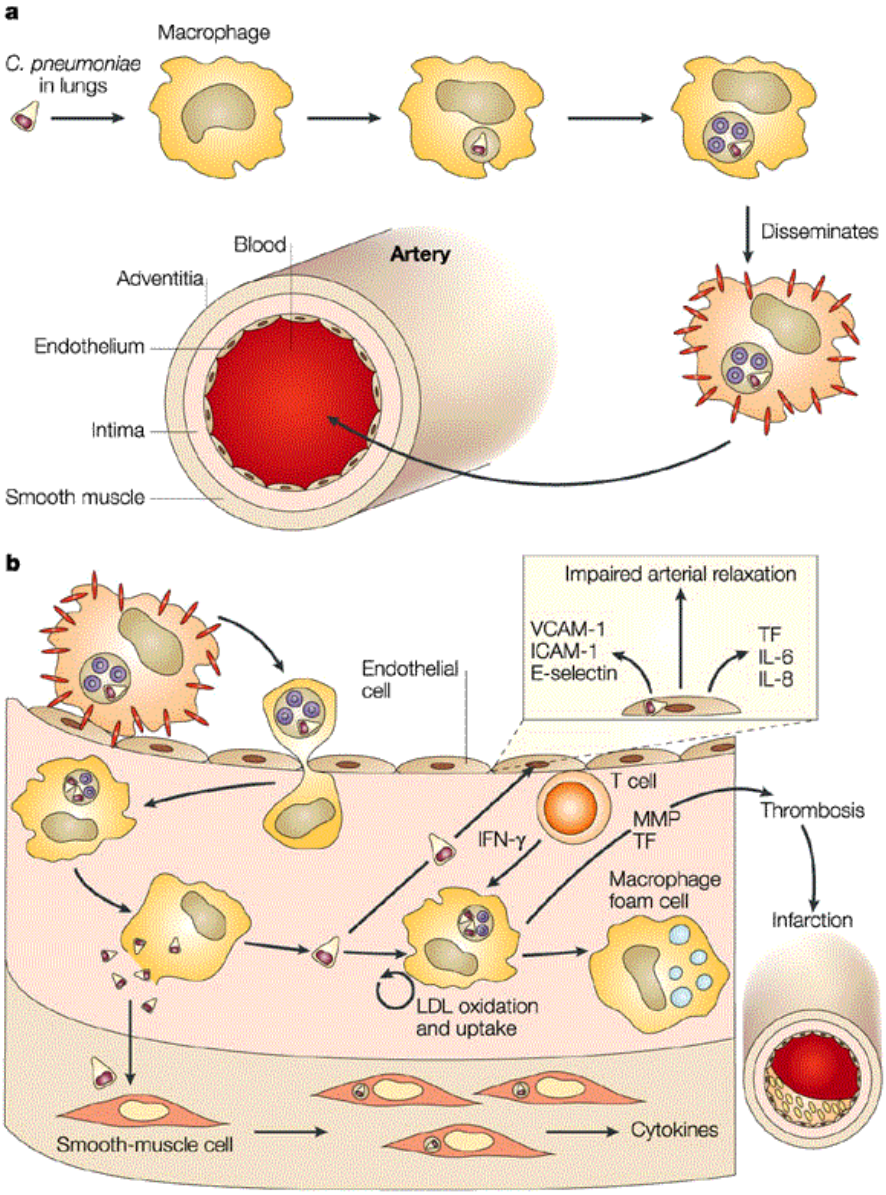


Figure 5: Chlamydia pneumoniae infection as initial step of inflammation provoking atherosclerosis (reprinted from reference [104]). a) ***The EB of the pathogen is endocytosed by a monocyte in an infected lung and differentiates into RBs. Such an inclusion in macrophages upregulates the expression of β2-integrin, which is located on the cell surface and is responsible for the attachement to endothelial cells (EC) [112].*** b) ***The infected monocytes gain entry through the endothelial cell layer into the intima of blood vessels, and EBs stimulate VSMC to express cytokines like PDGF [111]. EBs also migrate into ECs and upregulate the expression of the cell adhesion molecules VCAM-1, ICAM-1 and E-selectin [108, 113], together with an induction of TF and the pro-inflammatory cytokines IL-6 and IL-8 [114]. T-cells and further monocytes migrate through the endothelial cell layer and promote the formation of foam cells by an uptake of oxLDL, resulting in atherosclerotic plaques [109]and final infarction.***

1.4.3 Treatment of *Chlamydia pneumoniae* infection and perspective

Although there exist approaches to antagonize acute *C. pneumoniae* infection using macrolide antibiotics, such as azithromycin and clarithromycin, or ansamycins like rifampicin and rifalazil [115-117], the infection showed recidivism after only a few months. For the medication of the latent infection, there is no efficient therapy available. Several *in vitro* and *in vivo* studies have demonstrated this evident problem, as an infection may still/again be recovered subsequently after long-term antibiotic treatment [118, 119]. Due to the formation of persistent chlamydial inclusions (ABs), which stay unaffected by antibiotics, *C. pneumoniae* is able to maintain the state of a chronic infection [101]. Therefore, the profiling for new compounds to cure and prevent *C. pneumoniae* infection is needed.

In the search for new strategies against chlamydial infection, plant polyphenols like catechins, flavonoids, phenolcarbonic acids and stilbenes are possible candidates [120]. These compounds can be found in fruits, vegetables and beverages like tea and red wine. Polyphenols are associated with positive effects on health, and several studies have shown influence of polyphenol intake on inflammation [121-123]. Epidemiologic studies have demonstrated a beneficial effect of flavonoids concerning CVDs [124, 125]. Tea polyphenols as well as red wine with its prominent component *trans*-resveratrol [126] have shown an inhibitory effect on *C. pneumoniae in vitro* [127]. The flavone luteolin demonstrated antichlamydial activity *in vivo,* and diminished the inflammatory response in mice [128]. Thus, a screening of polyphenol-rich sources for new anti-chlamydial lead structures appears promising.

1.5 Objective

1.5.1 eNOS and red wine

Since red wine was object of a large number of investigations, especially concerning its ability to decrease the risk of cardiovascular diseases, various *in vitro* and *in vivo* test systems have been established to measure the effects of red wine and its constituents. Atherosclerosis is associated to cardiovascular diseases, and constitutes the result of a crucial pathophysiological process of the vascular system, termed as endothelial dysfunction. It is characterized by a decreased availability to prevent oxidative stress, thrombosis and inflammation. A hallmark of a functional endothelial cell layer is the proper amount of permanently released endothelial NO, which is provided by the eNOS enzyme. Therefore, maintenance or even improvement of eNOS activity is requested in order to antagonize the development and progress of atherosclerosis as a consequence of endothelial dysfunction.

Previous studies have remarkably illustrated that red wine was able to beneficially influence the eNOS system using EA.hy926 endothelial cells after long-term incubation (24 hours) at 600 µg/ml of a red wine phenol extract (RWPE). Leikert et al. [71] showed an enhanced eNOS transcription rate of the eNOS gene, measuring the eNOS promotor activity. In addition, RWPE upregulated the eNOS protein level dose-dependently. Furthermore, the eNOS activity was significantly higher in response to RWPE, applying the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay. Finally, the increased release of NO was quantified with the 4,5-diaminoflurescein assay. According to these *in vitro* results with RWPE, *trans*-resveratrol was tested at naturally occurring concentrations (between 1 µM and 10 µM) as possible inducer of these effects. It only revealed an effect on eNOS promotor activity, stabilization of mRNA and eNOS enzyme expression, but neither on eNOS activity nor on released NO [69, 71, 72]. This indicates (an) alternative red wine polyphenol(s) to be responsible for eNOS enzyme activation and related release of bioavailable endothelial NO (see Figure 6).

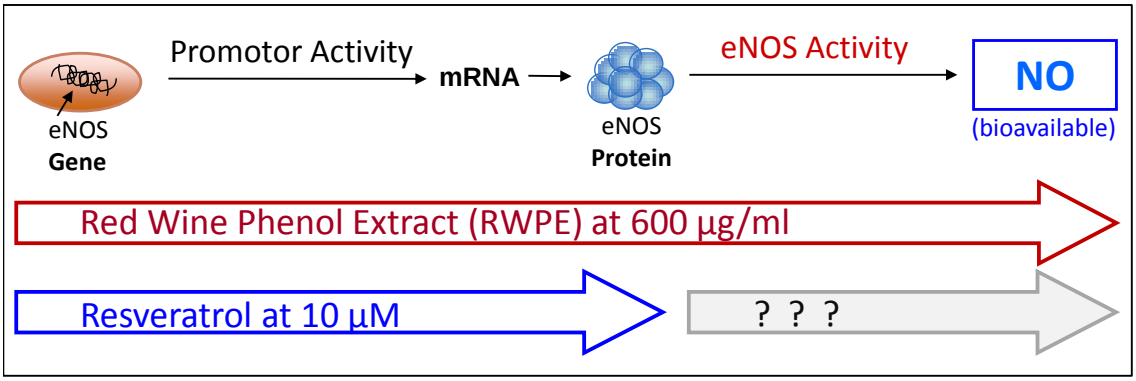


Figure 6: RWPE is mediating a stimulatory effect on the eNOS system at 600 µg/ml after long-term incubation, while trans-resveratrol is not the only activating red wine constituent.

The impact of previously investigated RWPE cannot be attributed exclusively to *trans*-resveratrol. Hence, one aim of this thesis was the activity guided isolation as a systematical approach to identify polyphenolic red wine constituent(s), particularly responsible for the enhancement of eNOS enzyme activity, and release of bioavailable NO. The identification of such compounds may contribute to the understanding of the eNOS enzyme as well as provide new strategies to face atherosclerosis and CVDs.

1.5.2 *Chlamydia pneumoniae* and red wine

C. pneumoniae is well-known to cause infections of the respiratory tract, but over the past years several studies proposed its contribution to the pathogenesis of atherosclerosis together with promoting precursory pathological mechanisms, such as inflammation and dysregulation of the vascular system [103, 104].

The release of endothelial nitric oxide, formed by eNOS, is a major function of the vasculature, and *C. pneumoniae* infection has been indicated to decrease eNOS expression *in vitro* [129, 130]. Recently, it is discussed to provoke endothelial dysfunction.

Plant polyphenols have shown influence counteracting inflammation as well as CVDs [48-52]. Consequently, red wine was studied together with *trans*-resveratrol, and both reduced *C. pneumoniae* infection [126].

Preliminary results using Austrian red wine, with relation to the investigations of this thesis, were consistent with the already mentioned antimicrobial activity. Based on these findings, the polyphenol-rich source red wine should be investigated in order to discover new antichlamydial agents (inhibiting growth and formation of inclusions).

For this purpose an activity guided fractionation of Austrian red wine was performed to identify further red wine constituents with inhibiting potential against *C. pneumoniae* infection. This study aims for the identification of new antichlamydial hit(s) to corroborate that an explanation of the French Paradox implicates various aspects. Furthermore, such a result would provide a new argument for atherosclerosis as a multifactorial disease.

2 MATERIALS AND METHODS

In this section, an overview of the applied materials, analytical and biological methods will be given. First the red wine samples for the investigation will be listed. Chapter 2.2 deals with the extraction, fractionation and isolation processes, followed by a brief introduction into the analytical methods in chapter 2.3. Chapter 2.4 delivers information on the cell culture, chapter 2.5 describes the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay, which is the main method used to measure the influence of red wine compound on the eNOS. Additionally, a modification adding catalase before stimulation is mentioned. The measurement of the released NO by a fluorescence-based assay (DAF-FM) is the subject of chapter 2.6. Chapter 2.7 presents a western blotting method to detect phosphorylation of eNOS regulatory domain Ser^{1177}. Section 2.8 describes methods regarding *Chlamydia pneumoniae* inhibition. Statistical analysis used for the data evaluation is mentioned in chapter 2.9. The applied compounds are listed in 2.10.

2.1 Samples

2.1.1 Austrian red wine samples

Sixty samples of Austrian red wine, grown and produced in various typical wine regions (Figure 7), were provided by the Federal College and Research Institute for Viticulture and Pomology (Klosterneuburg, Lower Austria; Head of Chemistry/Biology: Dr. R. Eder).

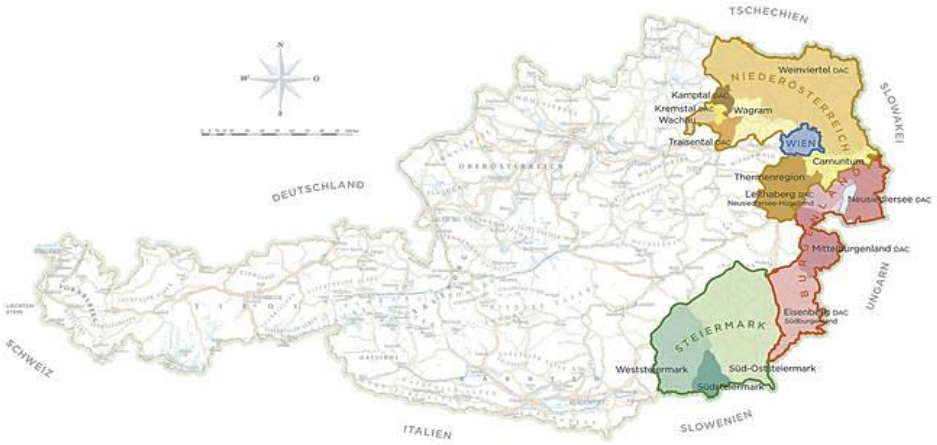


Figure 7: Typical wine regions of Austria (Österreich Wein Marketing GmbH (ÖWM); access: 2013/01/16. http://www.oesterreichwein.at/unser-wein/weinbaugebiete/).

This large batch of red wines provided quantification data of main phenolic constituents (see Table 3) for the systematic approach to elucidate eNOS-activating compounds using an activity guided fractionation. *Table 1* gives an overview on the distribution of the red wine samples according to variety, whereas *Table 2* shows the number of samples per region of origin.

Variety	Number
Pinot Noir (PN)	4
Zweigelt (ZW)	20
Sankt Laurent (SL)	7
Blaufränkisch (BK)	16
Lagrein (LG)	1
Blauburger (BB)	4
Blauer Portugieser (BP)	2
Cabernet Sauvignon (CS)	3
Merlot (ME)	3
Total	60

Table 1: Number of samples according to the region of origin

Origin	Number
Wien	20
Carnuntum	10
Mittelburgenland	13
Thermenregion	3
NÖ	6
Wagram	2
Burgenland	1
OÖ	1
Südtirol	1
Wachau	1
Kremstal	1
Neusiedlersee	1
Total	60

Table 2: Sample composition according to origin

In the following Table 3, quantitative data of fifteen polyphenols and three groups of phenols concerning the sixty Austrian red wine samples are illustrated. All samples were compared regarding their phenolic composition and eight red wines, which were outstanding regarding the content of compounds, were selected for the initial fractionation step and eNOS activity testing as described in section 3.1.1.

Approval number	Variety, Vintage and Origin	Gallic acid	Caftaric acid	c-Coutaric acid	t-Coutaric acid	Tyro-sol	Cate-chin	Epicate-chin	Caffeic acid	p-Coumaric acid	Fertaric acid	Ferulic acid	t-Piceid	c-Piceid	t-Res-veratrol	c-Res-veratrol	Res-veratrol (total)	Antho-cyanins (total)	Phenols (total)
		mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	g/l
K 604/06	ZW 05 Carnuntum	66.4	6.4	0.6	1.8	22.8	95.8	128.5	74.8	9.1	0.9	2.6	1.22	0.77	2.63	1.61	6.2	328	1.91
K 608/06	ZW 05 Carnuntum	133.9	114.3	3.2	20.2	30.2	69.3	99.0	13.1	3.1	4.0	1.1	1.34	1.16	1.40	1.40	5.3	194	1.59
K 624/06	ZW 05 NÖ	48.6	93.2	3.7	19.4	34.7	92.3	117.2	2.7	0.8	4.1	0.4	0.93	0.98	0.79	0.41	3.1	188	1.43
K 635/06	BLFRK 04 Wien	31.2	46.6	1.5	8.9	46.2	46.4	46.3	23.9	3.3	4.1	0.9	0.80	0.87	0.63	0.65	3.0	133	1.08
K 636/06	SL 04 Wien	32.7	13.9	0.9	4.2	41.5	60.3	36.0	27.9	7.5	2.3	1.5	0.12	0.14	0.84	0.60	1.7	151	1.04
K 657/06	SL 04 Wachau	32.9	14.9	1.3	3.0	9.6	38.4	31.0	3.2	1.9	2.3	0.7	0.14	0.20	0.13	0.15	0.6	26	0.51
K 684/06	ZW 05 Wien	25.5	51.5	3.3	11.8	17.8	38.7	38.0	19.9	4.7	2.7	0.8	0.30	0.30	0.85	0.78	2.2	128	0.60
K 719/06	ZW 05 NÖ	48.7	54.6	3.7	12.7	15.8	77.5	82.9	4.3	2.1	2.8	0.4	0.48	0.53	0.55	0.50	2.1	63	1.06
K 747/06	BB 03 Wien	22.8	103.5	2.6	23.6	36.4	23.3	41.5	3.0	1.2	7.2	0.8	0.72	0.78	0.40	0.39	2.3	91	1.09
K 790/06	ZW 05 Wien	56.8	96.3	3.7	16.2	26.8	67.5	106.2	4.9	0.9	3.6	0.6	0.68	0.69	0.88	1.19	3.4	148	1.69
K 802/06	ME 05 Carnuntum	42.8	94.4	5.6	25.1	18.4	93.6	115.2	2.9	1.6	3.1	0.8	2.73	4.73	3.18	2.01	12.7	275	1.82
K 817/06	ME 04 Wien	32.3	21.5	1.2	5.4	30.2	38.7	46.5	15.9	2.8	2.2	0.5	0.19	0.24	0.18	0.60	1.2	47	1.12
K 845/06	ZW 05 Carnuntum	54.6	90.1	2.5	18.0	24.5	63.8	99.9	27.8	3.5	3.6	0.6	1.14	1.05	2.07	1.95	6.2	242	1.70
K 847/06	ZW 04 Wien	37.0	48.6	2.1	11.0	31.1	37.0	53.4	9.4	4.5	3.4	0.2	0.42	0.41	0.34	0.61	1.8	91	0.86
K 860/06	ZW 05 Wien	85.5	9.7	0.9	2.7	12.6	101.6	113.1	29.9	4.8	1.0	2.0	0.33	0.37	0.37	0.70	1.8	98	1.40
K 861/06	ZW 05 Carnuntum	142.7	73.2	3.2	14.7	25.6	61.8	87.5	7.9	3.0	3.7	0.4	0.74	0.49	0.78	0.85	2.9	80	1.40
K 882/06	ME 04 Wien	86.7	35.9	2.8	9.8	24.4	77.4	52.6	22.8	4.2	2.1	0.9	0.97	1.67	0.47	0.89	4.0	22	0.97
K 889/06	PN 04 Wien	48.8	76.0	4.6	20.8	22.1	95.6	71.5	3.9	2.2	4.2	0.5	0.49	0.45	1.28	0.77	3.0	69	1.08
K 890/06	ZW 04 Wien	59.9	78.0	4.4	22.3	19.6	113.8	88.5	10.8	4.5	3.0	0.7	1.13	0.89	2.88	1.03	5.9	75	1.49
K 891/06	ZW 04 Wien	67.4	95.9	3.9	20.4	31.2	61.8	87.8	4.6	1.6	3.2	0.4	0.46	0.24	0.68	0.35	1.7	89	1.59
K 896/06	SL 05 Thermenregion	54.1	15.2	0.8	4.1	33.7	84.2	55.8	53.8	11.9	1.5	2.0	0.66	0.90	1.81	1.10	4.5	300	1.08
K 897/06	SL 05 Thermenregion	51.4	19.9	1.7	4.8	28.6	86.9	57.8	50.5	10.9	2.0	2.3	0.62	0.83	2.40	1.17	5.0	404	1.13
K 898/06	BLFRK 05 Thermenregion	85.0	130.2	4.0	18.6	15.3	131.3	178.5	9.3	2.6	4.7	0.6	0.40	0.33	2.57	1.30	4.6	239	1.58

Table 3: Quantitative data of the sixty Austrian red wine samples (generated from HPLC-analyses performed by the Federal College and Research Institute for Viticulture and Pomology, Klosterneuburg)

Approval number	Variety, Vintage and Origin	Gallic acid	Caftaric acid	c-Coutaric acid	t-Coutaric acid	Tyro-sol	Cate-chin	Epicate-chin	Caffeic acid	p-Coumaric acid	Fertaric acid	Ferulic acid	t-Piceid	c-Piceid	t-Res-veratrol	c-Res-veratrol	Res-veratrol (total)	Antho-cyanins (total)	Phenols (total)
		mg/l	mg/l	mg/l	Mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	g/l
Cp 3971	PN 04 Wien	40.0	56.6	8.4	14.4	18.7	73.3	42.6	3.5	2.1	2.4	1.2	0.76	0.66	2.09	1.09	4.6	63	1.09
Cp 3973	LA 04 Südtirol	19.7	29.3	2.3	7.8	49.7	27.0	20.0	11.1	4.8	0.9	1.3	2.03	2.46	2.44	1.18	8.1	124	1.51
Cp 3974	SL 05 Wien	24.1	25.9	1.5	5.0	33.9	37.8	15.5	19.7	4.5	2.1	1.0	0.67	1.06	0.80	0.78	3.3	158	1.09
Cp 3977	PN 05 Carnuntum	22.7	31.4	6.7	7.4	29.2	138.4	61.8	7.2	1.8	1.5	1.1	0.43	0.81	1.09	0.98	3.3	155	0.87
Cp 3978	ZW 06 Carnuntum	27,3	46.2	7.9	9.6	16.0	48.9	35.6	22.5	4.3	2.2	1.4	1.04	1.11	1.58	1.96	5.7	232	1.22
Cp 3979	PN 04 Wien	23.3	44.7	6.8	11.5	21.6	130.9	57.3	21.0	3.4	1.1	1.0	0.22	0.21	1.09	2.72	4.2	41	1.40
Cp 7433	BLFRK 04 Mittelburgenland	78.4	95.2	3.2	17.7	24.5	75.5	49.9	25.7	4.0	4.3	1.3	1.09	0.83	1.29	0.97	4.2	107	1.60
Cp 7434	BLFRK 04 Mittelburgenland	44.7	190.9	5.7	34.6	36.5	86.6	46.2	7.6	4.3	5.7	1.4	1.09	0.56	3.65	1.93	7.2	133	1.31
Cp 7435	BLFRK 05 Mittelburgenland	41.8	138.5	4.0	23.1	36.1	89.0	62.7	20.2	5.6	5.4	1.5	0.94	0.48	2.93	1.15	5.5	215	1.10
Cp 7436	BLFRK 05 Mittelburgenland	49.5	161.6	4.9	29.5	28.6	81.9	47.8	12.5	2.6	5.6	1.0	1.26	0.77	2.72	1.30	6.1	78	1.25
Cp 7437	BLFRK 05 Mittelburgenland	49.9	136.2	3.9	24.0	37.6	109.6	92.5	37.7	7.4	5.4	1.8	0.00	0.00	4.48	2.39	6.9	269	1.34
Cp 7438	BLFRK 04 Mittelburgenland	45.9	153.4	3.8	29.8	46.4	79.1	39.0	37.7	7.1	5.5	1.2	0.08	0.00	3.60	1.21	4.9	171	1.51
Cp 7439	BLFRK 05 Mittelburgenland	119.6	33.3	2.3	7.7	36.0	156.2	100.3	18.6	4.0	3.2	1.3	0.83	1.01	0.81	0.70	3.4	161	1.58
Cp 7440	BLFRK 05 Mittelburgenland	77.3	102.9	3.7	21.4	36.3	165.6	133.9	24.3	4.1	4.6	1.0	0.96	0.54	2.41	1.12	5.0	185	1.54
Cp 7441	BLFRK 05 Mittelburgenland	65.0	175.1	5.3	30.0	45.5	124.3	85.3	7.1	1.6	6.7	1.0	1.00	0.42	2.77	1.66	5.9	273	1.48
Cp 7442	BLFRK 05 Mittelburgenland	60.3	93.2	3.2	20.6	24.2	109.5	61.7	33.6	7.5	4.8	2.0	1.49	0.78	2.63	0.88	5.8	143	1.55
Cp 7443	BLFRK 05 Mittelburgenland	85.2	68.8	3.2	16.8	39.4	131.0	81.6	17.4	2.4	3.5	0.9	1.22	0.77	1.52	0.62	4.1	105	1.77
Cp 7444	BLFRK 05 Mittelburgenland	38.6	96.4	3.8	18.3	27.6	119.9	82.8	12.7	4.1	4.5	1.4	0.18	0.16	2.06	1.04	3.4	123	1.12
Cp 7445	BLFRK 04 Mittelburgenland	70.8	121.3	4.3	27.7	27.7	117.7	72.7	8.1	3.0	4.8	1.4	0.37	0.34	1.57	0.73	3.0	98	1.67
K 600/06	ZW 05 Kremstal	57.5	90.1	3.7	16.8	23.3	70.6	91.1	5.2	1.0	2.8	0.3	0.96	1.28	0.61	0.60	3.5	109	1.37

Approval number	Variety, Vintage and Origin	Gallic acid	Caftaric acid	c-Coutaric acid	t-Coutaric acid	Tyro-sol	Cate-chin	Epicate-chin	Caffeic acid	p-Coumaric acid	Fertaric acid	Ferulic acid	t-Piceid	c-Piceid	t-Res-veratrol	c-Res-veratrol	Res-veratrol (total)	Antho-cyanins (total)	Phenols (total)
		mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	g/l
K 901/06	ZW 05 Burgenland	54.2	72.8	2.6	15.6	30.0	83.6	124.5	31.3	4.7	3.1	0.9	1.74	1.71	1.83	0.75	6.0	348	1.52
K 902/06	BLFRK 05 Carnuntum	48.2	122.7	4.0	21.5	22.8	93.7	110.7	16.8	2.7	5.0	0.8	1.89	1.76	3.08	1.74	8.5	269	1.62
K 908/06	SL 04 Carnuntum	26.0	68.4	3.3	15.6	23.3	69.1	42.2	20.1	5.7	2.8	1.6	0.24	0.27	1.57	1.37	3.5	263	1.72
K 932/06	ZW 04 Wien	49.8	59.4	2.5	12.5	21.4	21.8	41.5	6.4	1.9	3.2	0.6	0.81	0.89	0.63	0.58	2.9	49	1.02
K 936/06	BB 05 Carnuntum	42.6	45.3	3.7	11.8	13.7	87.8	75.6	9.8	3.0	3.1	0.6	0.61	0.64	1.10	1.24	3.6	143	0.86
K 940/06	CS 04 Neusiedlersee	30.1	12.6	1.3	3.5	34.1	52.3	82.2	29.5	4.2	0.5	0.9	0.13	0.17	1.88	2.04	4.2	132	1.50
K 968/06	BB 04 Wien	41.1	60.7	2.7	10.8	26.0	32.2	36.9	4.9	2.2	5.2	0.9	0.19	0.12	0.30	0.55	1.2	74	0.84
K 1006/06	BP 05 NÖ	32.2	62.7	4.2	14.7	27.6	53.7	57.3	2.4	2.0	4.0	0.5	0.42	0.44	0.80	0.38	2.0	147	0.83
K 1018/06	CS 05 NÖ	60.5	75.1	4.9	17.6	10.2	62.1	88.7	8.1	2.0	3.2	0.9	0.59	0.47	0.84	0.68	2.6	97	1.55
K 1019/06	BP 05 NÖ	27.6	42.7	3.2	10.1	31.2	52.9	63.6	6.2	3.1	3.9	0.7	0.18	0.22	0.28	0.53	1.2	136	0.90
K 1026/06	ZW 05 Donauland	74.8	134.8	5.2	28.8	54.4	34.4	38.6	3.1	1.0	3.7	0.4	0.84	0.97	0.93	0.92	3.7	345	1.07
K 1027/06	ZW 04 Donauland	21.4	89.8	3.9	17.0	19.4	25.3	26.2	15.7	2.7	3.3	0.5	0.51	0.40	0.98	0.52	2.4	63	0.64
K 1031/06	SL 04 NÖ	14.8	44.7	3.1	8.3	29.5	36.7	13.2	8.9	6.8	2.5	0.9	0.00	0.00	0.66	1.19	1.9	78	0.80
K 1040/06	CS 04 OÖ	61.0	22.0	5.5	10.5	18.6	62.6	122.8	3.8	3.0	2.7	1.0	2.27	2.48	2.43	1.59	8.8	127	0.95
K 1059/06	ZW 05 NÖ	35.8	70.7	3.9	15.3	17.6	49.7	60.1	4.8	2.9	3.9	1.1	0.26	0.22	0.72	0.29	1.5	176	0.72
K 1062/06	ZW 05 Wien	28.6	75.7	3.6	16.1	52.3	36.7	55.7	11.9	2.8	3.3	0.6	0.86	1.35	0.66	0.55	3.4	216	0.97
K 1082/06	ZW 04 Wien	25.3	88.5	3.8	21.4	42.4	26.9	33.8	10.2	2.3	3.8	1.2	0.87	1.27	1.11	0.70	4.0	101	0.91
K 1083/06	BB 04 Wien	27.1	47.2	1.9	10.9	45.3	32.6	45.8	19.1	3.1	4.6	1.3	0.87	1.23	0.68	0.75	3.5	237	0.93

2.1.2 Red wine sample for the continued fractionation

After three Test Series (chapters 3.1.1, 3.1.2, 3.1.3) with the eight selected red wine samples have been carried out, it became obvious, that higher amounts of red wine were required for the assay. Hence, two new red wine samples, that were available in large amounts, were obtained from our cooperation partner, the Federal College and Research Institute for Viticulture and Pomology. We got two red wines of the same kind, called “Burgweingartl” (BWG) (vintage 2007 and 2008), which consisted mainly of the variety Blaufränkisch. These two samples were directly compared concerning their influence on the eNOS together with the most promising red wine of the Test Series investigations (Blaufränkisch Klosterneuburg 2005, see sections 3.1.3 and 3.1.4). The eNOS activity profiling was proceeded with the BWG of 2007 (chapter 3.1.4).

2.1.3 Vinification sample

The mash of Merlot grapes, which was used to investigate the influence of the fermentation process on the ability of the wine to modulate the activity of eNOS enzyme, was kindly provided by the winemaker Birgit Wiederstein (Göttlesbrunn, Lower Austria) in September 2007. The vinification was performed at the Department of Pharmacognosy based on the instructions of the winemaker. A sample aliquot was taken every three days to monitor the progress of fermentation. The process was completed after fifteen days and the vinification data were recorded in a fermentation protocol illustrated in chapter 3.1.8.

2.1.4 White wine sample

A typical Austrian white wine was also tested for the effect on eNOS. The “Grüner Veltliner 2006” (GV) was vinificated and bottled at the Federal College and Research Institute for Viticulture and Pomology and generously provided for research (section 3.1.11).

2.1.5 Barrique and steel cask red wine sample

To investigate a possible influence of the storage conditions on the enhancement of eNOS activity by wine, a Merlot 2007 red wine sample was drawn by winemaker Birgit Wiederstein (Göttlesbrunn, Lower Austria) in December 2007 just before transfer of the wine to either a barrique cask or a steel cask. A second sample was received in June 2008 shortly before the bottling of the wine. Details are figured in chapter 3.1.13.

2.1.6 Red wine samples for testing antichlamydial activity

For the investigations on the anti-chlamydial effect of red wine, which was done in cooperation with Prof. Pia Vuorela (Åbo Akademi University, Turku, Finland) and Dr. Olli Salin (University of Umeå, Sweden) two samples out of the sixty Austrian red wines (chapter 2.1.1), that have shown the best results in the Second Test Series (see section 3.1.2.2) were chosen for an activity guided fractionation. Those wines were the Blaufränkisch Klosterneuburg 2005 (BK) and the Zweigelt Carnuntum 2005 (ZC). Further details are given in chapter 3.2.

2.2 Extraction, fractionation and isolation

2.2.1 Preparation of the dealcoholized red wine concentrate (DRWC)

The first fractionation step included eight selected red wines for the First Test Series (chapter 3.1.1.1) of the eNOS activity guided fractionation. The samples were prepared for column chromatography by a tenfold reduction of red wine to get the compounds concentrated on the top of the column. The ethanol was almost quantitatively removed under reduced pressure at a temperature of 40 °C, and the obtained dealcoholized red wine concentrate (DRWC) of each red wine was transferred to the columns.

Besides the following fractionation, these DRWCs were also directly assayed for their influence on the eNOS enzyme activity (see chapter 3.1.1.3).

2.2.2 Column chromatography (CC)

The glass columns used for the First Test Series to separate the DRWC into the first eluate (FE) and the red wine phenol extract (RWPE) had a length of 100 cm, a diameter of 3 cm, a solvent reservoir of 2000 ml, and were filled with 150 g of stationary phase (weight before conditioning). The column for a further fractionation with another red wine sample (BWG 07) (see chapter 2.1.2 and 3.1.4) was 70 cm in length with 8 cm diameter and a reservoir of 3000 ml. It was filled with 1500 g of stationary phase (weight before conditioning). For both columns, the stationary phase was a polystyrene DIAION® H_20 material with a particle size of 150 µm – 800 µm (Sigma-Aldrich, Steinheim, Germany). It was first washed subsequently with methanol and distilled water and then suspended in methanol to fill the glass column (because a suspension in the starting eluent causes floating of the polystyrene material). After transferring the polystyrene suspension – under stirring or alternatively degassing of the solvent with helium – into the column and topping with purified sea sand, the column was able to get equilibrated with the mobile phase. The starting eluent of this simple two-step gradient was 10 % aqueous ethanol (v/v) solution. The second eluent in use was 90 % aqueous ethanol (v/v). This method was slightly adapted from literature [131]. The twentyfold sample volume of ethanol 10 % (v/v) was used to elute fruit acids, glycerol and sugars as FE. The RWPE was eluted with the tenfold sample volume of ethanol 90 % (v/v). Both fractions were lyophilized before further fractionation and eNOS activity testing. The total amounts of the dried extracts (FE + RWPE) of the applied red wine samples varied from 2.5 g to 3.5 g, whereby FE yielded about 90 % and RWPE approximately 10 % of the total amount. Further details and a scheme of CC are given in section 3.1.1.2.

2.2.3 Liquid-liquid partition (LLP)

The liquid-liquid partition is a simple method, which can be applied to divide compounds between two immiscible solvents depending on their polarity. For this approach, H_2O and ethyl acetate were used as phases and the resulting fractions were

defined as polar fraction (PF; H_2O-phase) and apolar fraction (AF; ethyl acetate-phase). The lyophilized, powdered RWPE was dissolved in a minimum of 70 % ethanol (v/v), and diluted 1:400 (w/v) with H_2O. Then, the partition was performed three consecutive times with 200 ml of ethyl acetate. The PF and the AF were freeze dried to prevent degradation of labile compounds (the ethyl acetate of the AF had to be evaporated, dissolved in a minimum of 70 % ethanol (v/v), and diluted with H_2O before freeze drying).

The PF yielded two thirds of the weight relatively to the RWPE, while the AF was the complementary third of the previous fractions weight, as illustrated in Figure 17.

2.2.4 Solid phase extraction (SPE)

The solid phase extraction represents a simplified and more reproducible modification of CC, more precisely of vacuum liquid chromatography. Bond Elut® C18 cartridges (Varian, Harbor City, CA, USA) containing 5 g of stationary phase and a reservoir volume (RV) of 20 ml were used for the consecutive fractionation of the AF. A stepwise gradient with different ratios of methanol/H_2O was established to achieve the solid phase fractions 1 – 5 (SPF 1 – 5). A concentrate of AF (200 mg of the fraction dissolved in the lowest possible quantity of 70 % ethanol (v/v)) was loaded onto the conditioned C18 cartridge and was eluted with the different ratios of methanol/H_2O (1 %, 5 %, 10 %, 40 % and 70 % methanol). Four RV of each eluent were collected representing SPF 1 – 5, and dried on a rotary evaporator. Finally, the SPFs were redissolved in approximately 300 µl of 70 % ethanol (v/v), transferred into plastic tubes (2 ml volume) and dried again under reduced pressure in a SpeedVac (Heto® CT110/VR-1).

2.2.5 Semipreparative high performance liquid chromatography (HPLC)

Semipreparative HPLC was performed for the fractionation and isolation of bioactive compounds from the AF of two samples (BK 05 and BWG 07). The HPLC-system is described in more detail in chapter 2.3.1. Relevant methods are listed in Table 4. Separation of eNOS activating compound(s) was carried out on a semipreparative

Purospher® C18e column (250 x 10 mm, 5 µm) with method P1, whereas fractionation for the anti-chlamydial assays was performed with an analytical Aquasil® C18 column (250 x 4.6 mm, 5 µm) as stationary phase and method P2. The fractions were collected using timed-cut methods at a detection wavelength of 280 nm.

Semipreparative method	Stationary phase	Mobile phase	Flow rate (ml/min)	Oven temperature (°C)	Injection volume (concentration)
P1	Purospher® C18, 250 x 10 mm, 5 µm	Aq. formic acid (A)(pH 2.70) and acetonitrile (B), 10 – 22.6 % B in 63 min, 22.6 – 50 % B in 5 min	4.7	30	100 µl (50 mg/ml)
P2	Aquasil® C18, 250 x 4.6 mm, 5 µm	Aq. formic acid (A)(pH 2.70) and acetonitrile (B), 1 – 11 % B in 60 min	1.0	30	30 µl (20 mg/ml)

Table 4: Parameters of the semipreparative HPLC-methods.

2.3 Analytical methods

2.3.1 High performance liquid chromatography (HPLC)

HPLC was also employed for analytical measurements. Different stationary and mobile phases were used together with variations in flow and temperature to create optimized methods for qualitative and quantitative analyses (sections 3.1.2.3, 3.1.3.3, 3.1.5). HPLC-measurement was used for the fingerprint comparison of the red wine samples´ AF, for the quantification of substances, for comparing red wine fractions with pure compounds, to verify the stability of fractions and compounds and for the characterization and structure elucidation coupled to various detectors, such as DAD, ELSD, and MS. All HPLC methods have an integrated purge at 95 % of acetonitrile and a re-equilibration time of about four column volumes, which are not explicitly displayed in Table 5.

Analytical method	Stationary phase	Mobile phase	Flow rate (ml/min)	Oven temperature (°C)	Injection volume (concentration)
A1	Aquasil® C18, 250 x 4.6 mm, 5 µm	Aq. formic acid (A) (pH 2.70) and acetonitrile (B), 1 – 11 % B in 60 min, 11 – 43 % B in 40 min	1.0	30	10 µl (2 mg/ml)
A2	Luna Phenyl Hexyl®, 250 x 2 mm, 5 µm	Aq. formic acid (A)(pH 2.70) and acetonitrile (B), 10 % B for 20 min, 20 – 40 % B in 20 min	0.4	20	30 µl (pure wine)

Table 5: Parameters of the analytical HPLC methods.

Generally, the Shimadzu HPLC-system (Kyoto, Japan) consisted of a system controller (CBM-20A), a membrane degasser (DGU-20A5), a solvent delivery unit (LC-20AD), an autosampler (SIL-20AC HT), a column oven (CTO-20AC), a photodiode array detector (SPD-M20A), and a low temperature light scattering detector (ELSD-LT; 40 °C). HPLC data were acquired with the Shimadzu LCsolution software version 1.25. All solvents and modifiers were of HPLC grade quality. H_2O was of double distilled quality (ddH_2O).

2.3.2 High performance liquid chromatography – Mass spectrometry (HPLC/MS)

HPLC/MS analysis was accomplished using an Dionex Ultimate 3000 RSLC®-series-system (Germering, Germany), coupled to a Bruker Daltonics HCT mass spectrometer® (Bremen, Germany), equipped with a 3D quadrupole ion trap and an orthogonal electrospray ionization source (ESI). As mass spectrometry is an essential analytical method for the examination of structures and their molecular mass, it was used to elucidate the structures of eNOS-activating compounds and determination of related molecules via MS^2 experiments in the negative ion mode (section 3.1.5). LC/MS parameters are shown in Table 6 and Table 7.

HPLC-method for MS	Stationary phase	Mobile phase	Flow rate (ml/min)	Oven temperature (°C)	Injection volume (concentration)
A3	Aquasil® C18, 250 x 4.6 mm, 5 µm	Aq. formic acid (A) (pH 2.70) and acetonitrile (B), 1 – 31 % B in 180 min	1.0	20	10 µl (2 mg/ml)

Table 6: Parameters of the analytical HPLC-method used for the LC/MS analysis.

	Infusion	Ion source	Mass analysator	Modus
MS-parameters	HPLC-method A3 (Table 6)	Negative ESI mode	Ion trap	Ultra scan
	Software	**Ion trap**	**ESI-parameter**	**MS/MS-parameter**
	esquireControl Version 6.2 (Build 62.24) DataAnalysis Version 4.0 (Build 234)	Scan range: MS^1: 90 – 1100 m/z MS^2: 40 – 1100 m/z Averages: MS^1: 5 spectra MS^2: 3 spectra	Nebulizer: 50 psi Dry temperature: 365 °C Dry Gas: 10 l/min Voltage capillary: 4 kV	Isolation with: 4.0 Precursor Ions AutoMS: 3 Fragmentation amplitude: 1.0

Table 7: Mass spectrometry parameters for the LC/MS experiments.

2.4 Cell culture of the EA.hy926 endothelial cell line

2.4.1 Cell provenience

The human endothelial cell line EA.hy926 was kindly donated by Dr. C.-J. Edgell (University of North Carolina, Chapel Hill, USA). It was established in 1983 as a fusion between primary human umbilical vein endothelial cells (HUVEC) and the human lung carcinoma cell line A549/8 [132]. This hybridization represents the quality of HUVEC because of the expression of endothelium specific von Willebrand factor, and it is characterized as permanent endothelial cell line [133]. EA.hy926 cells are an established model for the investigation of endothelial cell functions. On the one hand the EA.hy926 cells grow faster in a less complex medium and have a much longer lifespan than HUVEC, on the other hand they have been studied intensively and are better defined than HUVEC, where the genetic variation of each donor might be different.

2.4.2 Media and supplements

Dulbecco´s Modified Eagles Medium (DMEM, Lonza) without phenol red (EA.hy926 cells express estrogen receptors which interfere with phenol red) and glutamine was used for the cultivation of EA.hy926 cells (storage at 4 °C). Following supplements for DMEM were added (final concentrations):

- 10 % heat-inactivated fetal bovine serum (FBS)
- HAT supplement (100 µM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine; Lonza)
- Streptomycin (100 µg/ml; Lonza), Benzylpenicillin (100 U/ml; Lonza)
- L-glutamine (0.29mg/ml; Lonza)

FBS (Invitrogen) was stored like the other supplements at -20 °C. FBS was heat-inactivated before usage. 500 ml FBS was thawed for 30 minutes in the water bath (37 °C) until the thawing was completed. Further on, compared to a reference bottle containing water and a thermometer, the temperature was increased up to 59 °C. Reaching 57 °C the water bath was set to 57 °C and FBS remained at this temperature for 30 minutes, was cooled down to room temperature and aliquots of 50 ml were prepared and stored. The concentration of the NOS substrate L-arginine in DMEM is 482 µM. DMEM does not contain ascorbic acid, which is used for the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay as positive control.

2.4.3 Culture

EA.hy926 cells were grown in 175 cm² polystyrene flasks, containing 30 ml of the culture medium and located in a humidified and 5 % CO_2 conditioned air incubator at 37 °C. The medium was changed every three to four days during cultivation. Cells were only used for experiments between passage 18 and 28 to maintain sensitivity and genetic uniformity. EA.hy926 cells were tested routinely for mycoplasmas using the MycoAlert® Mycoplasma Detection Kit (Lonza).

2.4.4 Passaging

Cells were passaged once a week after reaching confluence. All the solutions got tempered at 37 °C in a water bath before usage. The monolayer was washed with 30 ml PBS, and 6 ml of Trypsin/EDTA-solution (T/E) were added to the 175 cm² flask. After approximately three minutes in the incubator, the trypsinization was stopped by

adding 14 ml culture medium. An appropriate aliquot to reach the confluence within one week of cultivation was separately transferred to a new culture flask. The split ratio was about 1:20 depending on cell density and growth rate. Another aliquot of 0.5 ml was used for the cell counting and viability analysis (Vicell^{TM}, Beckman Coulter). The settings for the measurement of basic cell parameters of EA.hy926 cells are shown in Table 8.

Minimum diameter (µm)	10	Minimum circularity	0.4
Maximum diameter (µm)	30	Decluster degree	Low
Cell brightness (%)	85	Number of pictures	50
Cell sharpness	100	Aspirate cycles	1
Viable spot brightness (%)	75	Trypan blue mixing cycles	3
Viable spot area (%)	5		

Table 8: VicellTM settings used for counting of EA.hy926 cells.

2.4.5 Cell plating for experiments

For all experiments with EA.hy926 cells six-well plates were used. To reach confluence, cells were seeded at a density of 5 x 10^5 cells/well in 5 ml culture medium and grown for three to four days. Subsequently, the treatment with extracts, fractions and pure compounds was performed.

2.4.6 Thawing

A cryovial with about one million cells was taken out of the liquid nitrogen and thawed in a water bath at 37 °C for one minute until a thin liquid film covered the inner wall of the plastic tube. The suspension was poured out into 30 ml of culture medium and stored in a 75 cm² flask in the incubator for one day. The medium was replaced by 15 ml fresh medium and the three to four day period of feeding and passaging followed.

2.4.7 Solutions and buffers

The solutions used in the cell culture room got sterilized either by filtration (0.22 µm filter; Sarstedt) or by autoclavation (121 °C, 15 minutes).

Phosphate buffered salt solution: (autoclaved)		
Na_2HPO_4	8.0 mM	1.48 g
KH_2PO_4	1.5 mM	0.43 g
NaCl	160 mM	7.20 g
H_2O		ad 1000 ml
pH 7.4		

Table 9: PBS solution.

Trypsin/EDTA solution in PBS: (sterile-filtered)		
Trypsin	0.05 %	0.5 g
DTA	0.02 %	0.2 g
H_2O		ad 1000 ml

Table 10: Trypsin/EDTA solution.

2.5 [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay

2.5.1 Principle

This *in vitro* assay can be used for the quantification of the enzyme activity of endothelial nitric oxide synthase (eNOS). The substrate L-arginine for the enzymatic reaction of eNOS is converted via oxidation to L-citrulline and NO. By reason that NO is a very reactive molecule and the constitutive NO level, specifically biosynthesized by eNOS, is quite demanding to detect, the measurement of equimolar produced amounts of L-citrulline may serve as alternative to quantify NO indirectly. To carry out this assay, radiolabeled [^{14}C]L-arginine is used as substrate; the conversion product [^{14}C]L-citrulline represents the corresponding quantity of NO. The measured ratio of [^{14}C]L-citrulline compared to its substrate [^{14}C]L-arginine is defined as eNOS activity.

2.5.2 Experimental

The EA.hy926 endothelial cells were cultured three to four days in six-well plates until confluence was reached. Then, they were treated with extracts, fractions, and pure compounds for 24 hours (long-term stimulation) and harvested for the experiments. The medium was removed, and the cells got washed before conditioning with 1 ml of HEPES buffer (pH 7.4, 37 °C). Equilibration time in the incubation hood was 15 minutes at 37 °C. Cells were fed with 10 µl [^{14}C]L-arginine solution (0.33 µM, equate to 0.1 µCi per well) for ten minutes. Moreover, 1 µl of ionomycin (Ca^{2+}-Ionophor A23187, 1 mM in DMSO) was added for a final concentration of 1 µM, in order to increase the basal NO production rate of endothelial cells. Thus, a higher sensitivity of detection can be expected. After a period of exactly 15 minutes, the conversion of [^{14}C]L-arginine was stopped placing the 6-well plates on ice. Then, the cells were washed two times with 1 ml ice-cold PBS to remove the buffer solution, and with 800 µl iced ethanol 96 % (v/v) the cells were lysed. For 10 minutes the ice-cooled plates were left on the shaker at 130 rpm and after 5 minutes in the air incubator at 37 °C the lysates were shaken at room temperature at the same speed. 600 µl of the ethanol supernatant were transferred into a plastic tube, 900 µl of pure water were added to each well of the plates, and the suspensions were shaken again for 15 minutes. The residuals from the well plates were transferred into the same plastic tubes and their contents (cellular extracts) were dried under vacuum with a SpeedVac SPD 1010 (Thermo) depending on the quantity of samples, e.g. 5 hours at 45 °C for 24 samples. The dried pellets were suspended in 35 µl water/methanol (1:1; (v/v)) to extract the relevant amino acids. Additionally, the plastic tubes were shaken at 200 rpm for 5 minutes and vortexed for 20 seconds. The extracts were centrifuged for 45 minutes at 3000 rpm and 15 µl of each samples were spotted onto aminated TLC plates (Polygram SIL N-HR, Machery-Nagel). The mobile phase is a mixture of ddH_2O, chloroform (p.a.), methanol (HPLC quality) and saturated ammonium hydroxide (35 % (W/V))(p.a.) (2:1:9:4; (V/V/V/V)). The runtime to separate the two radiolabeled amino acids was 75 minutes. After drying the TLC plates, they were developed together with a photo-plate for at least 24 hours and could be detected with a phosphoimager (BAS-1800II, Fujifilm) as a positive

picture on the photo-plate termed autoradiograph. The resolution of the imager was 50 µm, and the software AIDA (raytest) was used for the densitometric quantification of [^{14}C]L-arginine (R_f 0.5) and [^{14}C]L-citrulline (R_f 0.8) spots. The eNOS activity was depicted as the percentage of [^{14}C]L-citrulline in relation to [^{14}C]L-arginine. The scheme below describes the crucial steps of the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay.

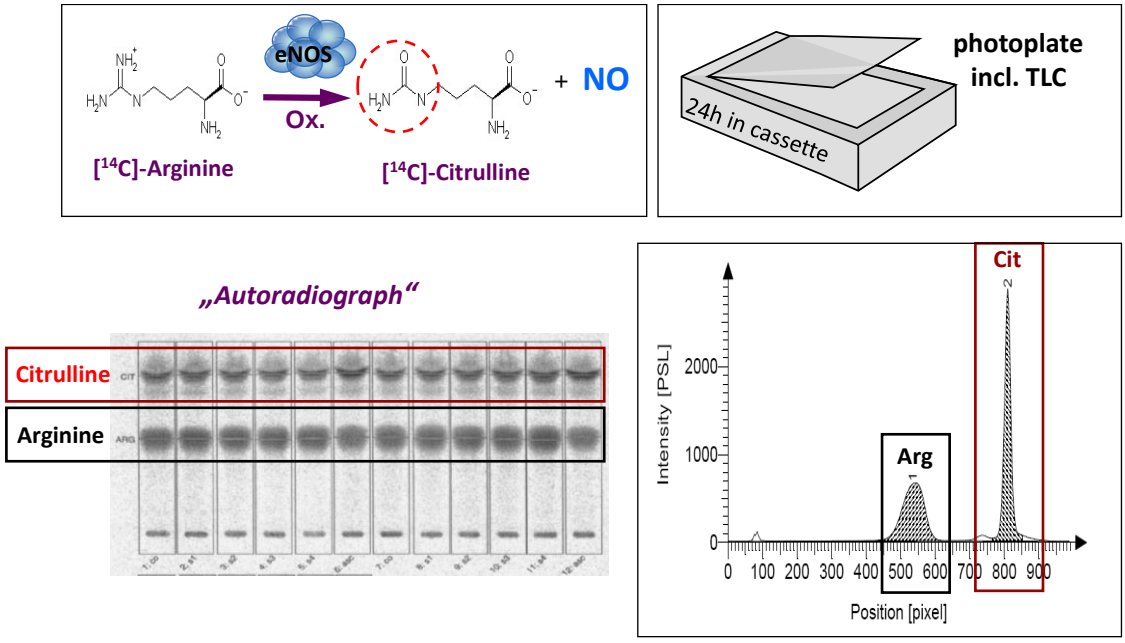


Figure 8: Scheme of the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay.

2.5.3 Inhibition of hydrogen peroxide induced eNOS activation (Catalase Assay)

To verify, that the measured eNOS activation performing the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was not negatively influenced by possibly generated hydrogen peroxide as a matter of stimulating endothelial cells with a large number of complex red wine polyphenolic constituents, this *in vitro* assay was carried out as well in a slightly modified way. 10 minutes before stimulation with RWPE and isolated active compound EG (see chapters 3.1.1.3 and 3.1.5) the bovine catalase (Sigma Aldrich) was added at a concentration of 100 U/ml to the cell medium preventing the

generation of hydrogen peroxide. The continuing procedure of the assay was similar to the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay.

2.5.4 Solutions and buffers

For the treatment of the EA.hy926 endothelial cells with [^{14}C]L-arginine they were previously washed and conditioned with 1 ml of HEPES buffer (pH 7.4, 37 °C). The tables below show the composition and dilution of these two solutions.

HEPES buffer pH 7.40, *stored at 4 °C*	
HEPES	0.476 g
NaCl	1.700 g
KCl	0.075 g
$MgSO_4$	0.049 g
$CaCl_2$	0.044 g
Glucose	0.360 g
dd H_2O	ad 200 ml

Table 11: HEPES buffer composition.

[^{14}C]L-arginine solution, *stored at 4 °C*
[^{14}C]L-arginine (New England Nuclear, UK; 313 Ci/mmol)
diluted 1:10 in water

Table 12: Dilution of [^{14}C]L-arginine.

2.6 3-Amino, 4-aminomethyl-2',7'-difluorofluorescein (DAF-FM) detection for the quantification of released endothelial NO

2.6.1 Principle

The physiological importance of NO is represented by the extra-endothelial levels of NO, released to the surrounding areas of endothelial cells, such as the lumen of blood vessels and the vascular smooth muscle cells. The diffusion of NO into the vasculature indicates the bioavailability level of NO, which is the intention to measure. Thus, additionally to the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay, which was used to measure the eNOS activity, the 3-Amino, 4-aminomethyl-2',7'-difluorofluorescein assay (DAF-FM) was employed to measure extracellular NO (chapter 3.1.7).

3-Amino, 4-aminomethyl-2',7'-difluorofluorescein (DAF-FM) is a fluorescent dye which is used to detect extracellular NO selectively, and hence does not interact with further reactive oxygen and nitrogen species like superoxide, hydrogen peroxide or peroxynitrite. As a consequence of possible interference with fluorescence influencing factors, such as bivalent cations (i.e. Mg^{2+} and Ca^{2+}) and a low pH, the system is stabilized with a defined buffer solution (section 2.6.4).

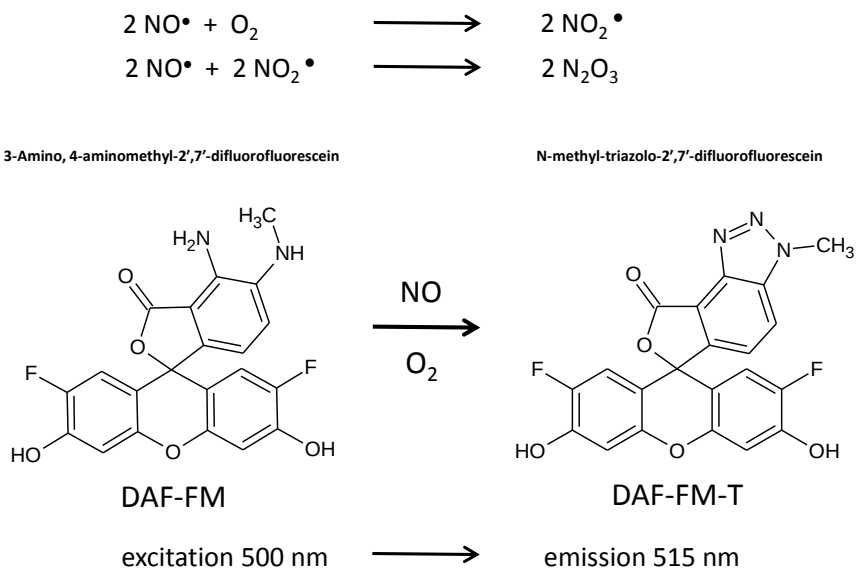


Figure 9: Stoichiometry of NO with oxygen; reaction of DAF-FM to DAF-FM-T in presence of NO and oxygen, measured at an emission of 515 nm.

DAF-FM is not directly involved in a reaction with NO, but in the presence of oxygen the formation of the reactive anhydride of nitrous acid, namely dinitrogen trioxide (N_2O_3) reacts with DAF-FM immediately as triazolofluorescein (DAF-FM-T), illustrated in Figure *9*. The emitted light is measured at 515 nm and represents the quantity of bioavailable NO. The stoichiometry of the reaction is 1:2 (DAF-FM:NO).

2.6.2 Experimental

The quantification of released NO from endothelial cells was performed with 3-Amino, 4-aminomethyl-2',7'-difluorofluorescein (DAF-FM), which is a NO-sensitive reagent. The EA.hy926 cells were seeded in 96-well plates at 2.5 x 10^4 cells/well (200 µl/well) and were stimulated with fractions or compounds after reaching confluence (approximately 72 hours). Cells were washed twice with PBS+ containing 100 µM L-arginine, and were equilibrated for ten minutes. Then, ionomycin (Ca^{2+}-Ionophor A23187) was added at a final concentration of 1 µM. DAF-FM had a concentration of 0.01 µM. The incubation time of the cells was one hour at 37 °C. For the determination of the background, samples were treated with L-NAME, an eNOS inhibitor, at 200 µM. The supernatant was transferred to a black 96-well plate and fluorescence was measured using a plate reader (Genios pro, Tecan; Austria) with an excitation wavelength of 500 nm and an emission wavelength of 515 nm. The resulting fluorescence values of the stimulated cells were normalized to their viability, which was detected by resazurin staining. The resazurin assay was performed in combination with the DAF-FM assay. The cells were stimulated with 0.1 mg/ml resazurin for 30 minutes and the fluorescence was quantified using a plate reader (Genios pro®, Tecan; Austria). The excitation wavelength was 535 nm, and the emission wavelength was 590 nm.

2.6.3 Limitations of the DAF-FM assay to measure released NO after treatment of endothelial cells with RWPE and other red wine fractions

The detection of NO release using the DAF-FM assay was performed to further elucidate the influence of eNOS modulators, besides supporting the results of the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay, which shows eNOS enzyme activity.

It was the intention to compare the initial red wine fractions, such as the RWPE and the consecutively obtained AF (apolar fraction) and PF (polar fraction), with isolated eNOS-activating compound(s). Surprisingly, after stimulation with RWPE, AF, and PF the response of the experiments to measure bioavailable NO did not match the expectations. The samples´ NO release did not differ from the control level when performing the DAF-FM assay. A controversial result of the later obtained NO release of an isolated eNOS-activating compound (see section 3.1.5) supported the assumption, that the fluorescence-based DAF-FM assay could not be employed for detecting the NO release of such a complex mixture, such as the RWPE or even AF or PF. Additionally, the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed to analyze the enzyme activity of the latter fractions, which indicated a significant effect (see chapters 3.1.1.3 and 3.1.2.2).

To find out, whether red wine fractions show interference and influence the response of the DAF-FM assay negatively, the positive control Mevastatin (3 µM; response in the DAF-FM assay is about ten- to twelvefold higher than control level) was co-stimulated with the AF and the PF, separately.

If the levels of AF plus Mevastatin and PF plus Mevastatin were identical to Mevastatin as positive control, these fractions would not be able to influence the DAF-FM assay, anyway. But, if the co-stimulated Mevastatin samples differed from their positive control significantly, an appropriate usage of this assay to measure the NO release of several fractions and extracts might not be possible.

As shown in Figure 10 below, the co-treated samples were not able to enhance the response comparably with mevastatin. The significantly reduced effect of mevastatin, combined with AF or PF, is therefore in line with the result of the pure AF and PF and

points out, that such fractions as AF and PF (and RWPE) could not be analyzed properly.

Reasons for that could be self-fluorescent compounds in these complex fractions or that AF and PF are interfering with the DAF-FM reagent because of their radical-scavenging properties. Finally, the DAF-FM assay to quantify the NO release has its limitations in respect to measuring extracts or fractions like the RWPE, the AF or the PF in contrast to pure compounds (Figure 43).

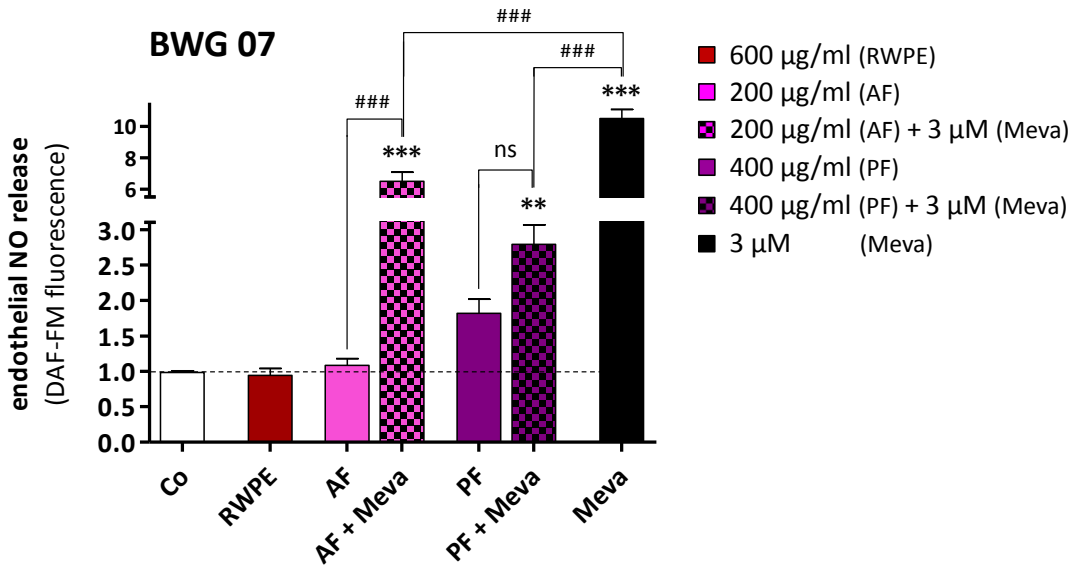


Figure 10: AF and PF of BWG 07 do not influence the endothelial NO release significantly, but show a decreasing effect combined with Mevastatin (positive control).

*Measurement of the endothelial NO release was carried out by incubation with the NO-sensitive fluorescent probe DAF-FM. EA.hy926 cells were treated with the listed concentrations for 24 hours. DAF-FM fluorescence was normalized to the cell count and to the activity of the control cells. Mevastatin (Meva, 3 µM) served as well as positive control. Control cells were treated with DMSO (0.1 %) (**, p < 0.01; ***, p < 0.001; ###, p < 0.001; ns, not significant) (Five replicates, mean ± SEM, n = 3 - 8, ANOVA/Bonferroni).*

2.6.4 Solutions and buffers

In this section, the used buffer and stock solutions for the DAF-FM assay to measure the extracellular NO are listed.

Aqueous PBS+ buffer pH 7.40, *stored at 4 °C*	
KCl	2.68 mM
NaCl	137 mM
Na_2HPO_4	8.10 mM
$MgCl_2$ x 6 H_2O	0.5 mM
$CaCl_2$ x 2 H_2O	0.68 mM
KH_2PO_4	1.47 mM

Table 13: PBS+ buffer composition.

PBS+Arg	100 mM L-arginine stock solution, diluted 1:1000 in PBS+
DAF-FM stock solution	5 mM DAF-FM stock solution, solved in PBS+
Ionomycin A23187 stock solution	10 mM A23187 stock, DMSO as solvent
L-NAME stock solution	1 mM solution in H_2O as stock

Table 14: Stock solutions for the DAF-FM assay.

2.7 Detection of serine^{1177} phosphorylation using Western blot

2.7.1.1 Principle

For the quantification of proteins as well as for determination of phosphorylation levels at specific amino acid residues “Western Blotting” is the well-established method.

The cells get lysed directly on the plate(s). Lysis buffer is containing CHAPS to solve the eNOS quantitatively out of the matrix enforced by ultrasonication. Then, denaturation of the proteins was examined by boiling with 2-mercaptoethanol, and furthermore charging with SDS. The separation was carried out in a PAA gel using discontinuous electrophoresis and transferring to PVDF. After incubation with a specific primary antibody and a coupling reaction with a secondary antibody, horseradish peroxidase was added to detect the emitted light in an adequate buffer solution. The measured light is proportional to the quantity of the spotted protein.

2.7.1.2 Cell growth and stimulation

The experiments were performed in three parallel wells with EA.hy926 cells. 0.4×10^6 cells were plated on 6-well plates to reach confluence within four days. The cells were treated with 10 µM and 50 µM ethyl gallate or were left untreated for 48 h.

2.7.1.3 Quantification of the protein

The Bradford method was used to measure the concentration of protein. It is based on a bathochromic shift of absorbance of Coomassie brilliant blue G-250 from 465 nm to 595 nm. 5 µl of the cell lysates were diluted 1:20 with distilled water and a triplicate of each sample was transferred to 96-well plates including a dilution of BSA (absolute concentration from 2.5 to 25 mg/ml). After that, 190 µl of Bradford reagent (1:5 dilution of Roti®-Quant, Carl Roth GmbH) was added to each sample, the plates were shaken for five minutes at room temperature, and finally the absorbance at 595 nm was quantified with a Tecan Sunrise™ microplate reader.

2.7.1.4 Blotting

PAA gel (polyacrylamide) was polymerized using a 30 % solution of PAA with 0.8 % of bisacrylamide, reaching a final concentration of 10 % (Rotiphorese® Gel 30, Carl Roth GmbH). A Mini-Protean™ 3 Cell System (BIO-RAD) was used for the development. Each pocket of the gel was filled with an aliquot of 20 µg protein and the separation was performed at 30 mA for 75 minutes. The PVDF membrane (polyvinylidenedifluoride; Immuno-blot™, Bio-Rad) was conditioned with methanol and blotting buffer for one minute. The proteins moved over to the PVDF membrane at 100 V for 90 minutes in a Mini TransBlot™ Electrophoretic Transfer Cell System (Bio-Rad), and the membrane was blocked in 5 % fat-free milk powder, solved in TBS-T, for 60 minutes and washed three times for ten minutes with TBS-T. The membrane was developed with a specific primary antibody overnight at 4 °C. After washing three times with TBS-T, the membrane got incubated with a species-specific HRP-conjugated secondary antibody for two hours. The blot was triple-rinsed with TBS-T for ten minutes and finally once with distilled water. Actin was detected after five minutes in ECL-solution. The eNOS protein spots were envisioned with a luminescent image analyser (LAS-3000™, Fujifilm) and the relative amounts were indicated densitometrically using AIDA software (Raytest). Quantities of eNOS were normalized to the actin levels of the related lanes. The following Table 15 and Table 16 describe antibodies, buffers, gels and inhibitors, which served for the western blot analysis.

Target	Dilution	Solution in TBS-T (%)	Origin	Provider
eNOS	1:2500	-	Mouse	BD
Phospho-eNOS-Ser^{1177}	1:1000	5 % BSA	Rabbit	Cell Signaling
Actin	1:1000	-	Rabbit	Santa Cruz
Mouse IgG	1:1000	-	Goat	Update
Rabbit IgG	1:2500	5 % milk	Goat	Santa Cruz

Table 15: Antibodies for the western blot experiments.

eNOS lysis buffer, *freshly prepared prior to use*	
eNOS lysis buffer stock	910 µl
Complete™ protein inhibitor 25x	40 µl
Phosphatase inhibitor stock 20x	50 µl

eNOS lysis buffer stock pH 7.5, *stored at -20 °C*	
Tris-HCl	197.0 mg (50 mM)
CHAPS	308.0 mg (20 mM)
EDTA	3.9 mg (0.5 mM)
EGTA	4.7 mg (0.5 mM)
DTT	7.7 mg (2 mM)
Glutathion	53.7 mg (7 mM)
Glycerol	2.5 ml (10 % V/V)
ddH_2O	ad 25 ml

Phosphatase inhibitor cocktail 20 x, *stored at -20 °C*	
NaF	42.0 mg (0.2 M)
Na_3VO_4	36.8 mg (40 mM)
$Na_4P_2O_7$ x 10 H_20	223.0 mg (0.1 M)
ddH_2O	ad 5 ml

Complete™ protein inhibitors 25x
One tablet (Roche Diagnostics Germany) dissolved in 2 ml ddH_2O.

3 x sample buffer, *freshly prepared prior to use*	
WB sample buffer stock	850 µl
2-mercaptoethanol	150 µl

WB sample buffer stock	
Tris-HCl (0.5 M; pH 6.8)	37.5 ml
SDS	6.0 g
Glycerol	30 ml
Bromphenol blue	15.0 mg

Resolving gel (10 % PAA)	
Roti®-Phorese Gel 30	5 ml
ddH_2O	6.1 ml
Tris-HCl (1.5 M; pH 8.0)	3.75 ml
SDS 10 %	0.15 ml
TEMED	15 µl
Ammonium persulfate 10 %	75 µl

Stacking gel	
Roti®-Phorese Gel 30	1.7 ml
ddH_2O	7.0 ml
Tris-HCl (1.25 M; pH 6.8)	1.0 ml
SDS 10 %	0.1 ml
TEMED	20 µl
Ammonium persulfate 10 %	100 µl

5x Electrophoresis buffer, *stored at 4 °C*	
Tris-base	15 g
Glycine	72 g
SDS	5 g
ddH_2O	ad 1000 ml
5x Blotting buffer, *stored at 4 °C*	
Tris-base	15.2 g
Glycine	72.9 g
ddH_2O	ad 1000 ml
Blotting buffer, *stored at 4 °C*	
5x blotting buffer	200 ml
Methanol	200 ml
ddH_2O	ad 1000 ml
TBS-T pH 8.0, *stored at 4 °C*	
Tris-base	3.0 g
NaCl	11.1 g
Tween-20	1 ml
ddH_2O	ad 1000 ml
West-Dura ECL, *freshly prepared prior to use*	
Reagent 1	180 µl
Reagent 2	180 µl
ddH_2O	240 µl
Home-made ECL, *freshly prepared prior to use*	
ddH_2O	4.5 ml
Tris-base (1 M; pH 8.5)	0.5 ml
Luminol (0.25 M in DMSO)	25 µl
p-coumaric acid (90 mM in DMSO)	11 µl
H_2O_2 30 %	3 µl

Table 16: Buffers, inhibitors and gels for western blot experiments.

2.8 Approach for the C*hlamydia pneumoniae* investigations

2.8.1 HL cell line

The HL cell line (human lung) was generously provided by Prof. Pekka Saikku from the University of Oulu, Finland. HL cells were used as host cells for the infection with *Chlamydia pneumoniae* [120]. They were grown at 37 °C in an incubator with a condition of 5 % CO_2 and 95 % humidity in cell culture flasks (Greiner, bio-one, Frickenhausen, Germany) using RN-medium consisting of RPMI 1640 w/o L-glutamine

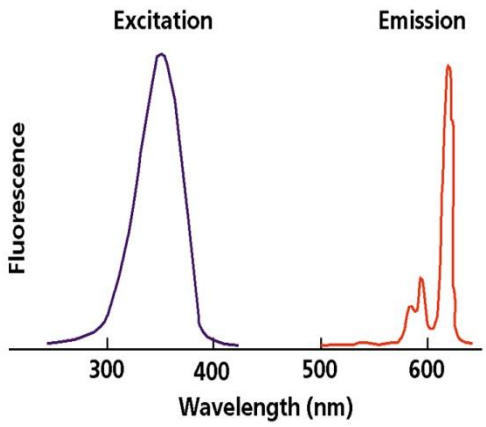
with 7.5 % of fetal bovine serum (FBS) (Biowhittaker, Lonza, Basel, Switzerland), 2 mM L-glutamine (Biowhittaker, Lonza, Basel, Switzerland) and 20 µg/ml of gentamycin (Fluka, Buchs, Switzerland).

The *Chlamydia pneumoniae* strain CWL-029 (American Type Culture Collection reference strain VR-1310) was grown and purified as described in Alvesalo et al. 2006 [134] for the experiments.

2.8.2 Time-resolved fluorometric immunoassay for growth inhibition measurement

The time-resolved fluorometric immune-assay (TR-FIA) is an approach to measure the bacterial growth inhibition due to a *C. pneumoniae* infection and hence, the anti-chlamydial effect of pure compounds, fractions and extracts was determined by TR-FIA. After reaching the confluence HL cells were washed with a 6.7 mM PBS buffer (Biowhittaker, Lonza, Basel, Switzerland) and moreover detached with 0.25 % trypsin (Biowhittaker, Lonza, Basel, Switzerland) in PBS. 0.6 x 10^5 cells/well were dispensed to the 96-well plate (Cellstar, Greiner, bio-one, Frickenhausen, Germany) in 200 µl of RN-medium and left for 24 h to attach. Furthermore, the medium was exchanged by RN-medium supplemented with cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) at 0.5 % and 0.2 multiplicity of infection (MOI) of *C. pneumoniae*. Plates were centrifuged at 550 x *g* for 60 minutes at room temperature and were kept for another 60 minutes at the incubator conditions. Then, the infective suspension and the non-infected medium were replaced by 200 µl/well. Six replicates and a control for each measured sample (extract, fraction and pure compound) were diluted to a concentration of 40 µg/ml in RN-medium with 0.5 % cycloheximide. After an incubation time of 70 hours the medium was removed, the HL cells were washed with 300 µl PBS and finally fixed with 300 µl methanol (Baker, Phillipsburg, USA) per well. The following day, the plates were labeled with assay buffer (PerkinElmer Finland, Turku, Finland), which contains europium-conjugated chlamydial antibody (100 ng/ml) (PerkinElmer Life and Analytical Sciences - Wallac, Turku, Finland). After 30 minutes of treatment at 37 °C the cells were washed six times with wash solution (PerkinElmer Finland, Turku, Finland) using a platewasher BW 50 (Biohit, Helsinki, Finland). To detach the europium for the

measurement of the emitted light, enhancement solution (pH < 1) was added to the wells and the plates were shaken for five minutes at low intensity on a plateshaker (Delfia Plateshaker, PerkinElmer Finland, Turku, Finland). The fluorescence was measured using a multilabel plate reader (Victor, PerkinElmer Finland, Turku, Finland) and got measured with a plate reader (Genios pro, Tecan; Austria) with an excitation wavelength of 340 nm and an emission wavelength of 615 nm. The growth inhibition of *C. pneumoniae* is represented by a decrease of the fluorophor´s signal intensity.



The red wine extracts were examined for autofluorescence and for label binding properties following the TR-FIA protocol above, without the infection and labeling to exclude interference with the assay.

Figure 11: Wavelength shift as principle of the time-resolved fluorometric assay to measure Chlamydia pneumoniae growth inhibition. access: 2014/03/02. **http://www.perkinelmer.com/pages/030/nbs/methods/time-resolved-fluorometry**

2.8.3 Immunofluorescence microscopy method to quantify inclusions

The immunofluorescence microscopy (IF) is carried out to detect *C. pneumoniae* inclusions after treatment with pure compounds, fractions and extracts. IF is based on fluorescein isothiocyanate-conjugated monoclonal murine antibody (Pathfinder® Chlamydia Culture Confirmation System (Bio-Rad, Hercules, USA)). It targets the lipopolysaccharide membrane of inclusions. The excitation wavelength is 480 nm and emission wavelength for the detection of inclusions is 510 nm.

For the reason that the eradication of *C. pneumoniae* is the bottleneck of curative therapy a remarkable reduction of formed inclusions is requested.

Confluent HL-cells were washed with PBS and detached with 0.25 % trypsin in PBS. 4×10^5 cells/ml were dispensed onto 24-well plates (Cellstar, Greiner, bio-one, Frickenhausen, Germany) with #1.5 coverslips (Menzel-Gläser, Braunschweig, Germany) in 1 ml of RN-medium and left to attach for 24 h. The medium was

exchanged by 200 µl of RN-medium supplemented with 0.5 % cycloheximide and 0.2 MOI (multiplicity of infection) of *C. pneumoniae*. Plates were centrifuged at 550 x *g* for 60 minutes at room temperature and stored in the incubator for further 60 minutes. After removal of the infective suspension, dilutions of pure compounds and red wine fractions were analyzed as duplicate or triplicate including a control for the dose response measurement. Then, the plates were incubated for 70 hours and the treatment was stopped by washing the cells with 1 ml of PBS. They got fixed with 1 ml methanol per well. The coverslips were removed from the wells and labeled with the Pathfinder® Chlamydia Culture Confirmation System according to the manufacturer´s instructions. The excess label was cleared away by dipping the coverslips one time into PBS and two times into deionized water (MilliQ). The coverslips were transferred to glass slides and analyzed by fluorescence microscopy with a magnification of 200. The quantity of chlamydial inclusions was the result of counting four defined areas of each coverslip, which added up to 8 to 12 observations per sample.

2.8.4 Host cell viability assay

All plates used for *in vitro* experiments were observed under a microscope for abnormalities before stimulation with compounds, prior to the fixing of the cells monolayer with methanol and before carrying out the viability determination.

The effect on host cell viability was employed with the commercial CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, USA). Seeding of the HL-cells on the 96-well plate was followed as close as possible to the TR-FIA procedure. The controls and the extracts or the pure compounds were added as dilution series after infection in triplicate. Measuring of adenosine triphosphate (ATP) content of cells was done after 70 hours treatment. To perform this measurement, the 200 µl of growth medium containing the stimuli was removed and 100 µl of fresh medium was added. Then, 100 µl of CellTiter-Glo® reagent was added, according to the manufacturer's instructions. Luminescence of oxyluciferin was determined with a Varioskan Flash multimode reader at 560 nm (Thermo Fischer Scientific, Vantaa, Finland).

2.9 Statistical analysis

Statistical analysis was accomplished using GraphPad PRISM™ software, Version 5.00 (GraphPad Software Inc.). One-way ANOVA with Bonferroni post test or t-test were done to compare multiple treatments (experiments) with the corresponding control. $P < 0.05$ was set for significance (CI). Graphs are illustrated as means ± SEM.

2.10 Reference compounds

Pure reference compounds were used for identification as well as for biological activity testings. Their purity and the providers are listed in Table 17.

Gallic acid	**99 %**	**Carl Roth GmbH, Karlsruhe, Germany**
Methyl gallate	**99 %**	**Sigma Aldrich, Steinheim, Germany**
Ethyl gallate	**99 %**	**Sigma Aldrich, Steinheim, Germany**
Propyl gallate	**99 %**	**Sigma Aldrich, Steinheim, Germany**
Butyl gallate	**98 %**	**ABCR, Karlsruhe, Germany**
Isopentyl gallate	**98 %**	**ABCR, Karlsruhe, Germany**
Octyl gallate	**99 %**	**Sigma Aldrich, Steinheim, Germany**
Lauryl gallate	**99 %**	**Sigma Aldrich, Steinheim, Germany**
Syringic acid	**98 %**	**Fluka Chemika, Buchs, Switzerland**
Coumaric acid	**98 %**	**Fluka Chemika, Buchs, Switzerland**
Caffeic acid	**99 %**	**Sigma Aldrich, Steinheim, Germany**
Tyrosol	**99 %**	**Sigma Aldrich, Steinheim, Germany**
Vanillic acid	**98 %**	**Fluka Chemika, Buchs, Switzerland**
p-OH-benzoic acid	**99 %**	**Sigma Aldrich, Steinheim, Germany**
Protocatechuic acid	**98 %**	**Carl Roth GmbH, Karlsruhe, Germany**
t-Resveratrol	**99 %**	**Sigma Aldrich, Steinheim, Germany**
c-Resveratrol	**99 %**	**Santa Cruz Biotechnology, Heidelberg, Germany**
(+)-Catechin	**98 %**	**Carl Roth GmbH, Karlsruhe, Germany**
(-)-Epicatechin	**98 %**	**Carl Roth GmbH, Karlsruhe, Germany**
Ascorbic acid	**99 %**	**Sigma Aldrich, Steinheim, Germany**
Mevastatin	**95 %**	**Sigma Aldrich, Steinheim, Germany**
Rifampicin	**99 %**	**Sigma Aldrich, Steinheim, Germany**
3-O-methyl gallate	**Purum**	**Isolated at the Dept. of Pharmacognosy, Vienna**

Table 17: Purity and providers of reference compounds.

3 RESULTS

This section will give the findings of the practical work performed for the thesis and is presented in two parts. The first chapter (3.1) deals with the activity guided fractionation of red wine samples to identify compounds indicating influence on the eNOS-system (activity of the eNOS enzyme and release of NO). Moreover, the importance of vinification on the eNOS activity is examined. In this context, various wine samples were compared for their content of a main eNOS-modulating compound. In section 3.2, the potential of red wine fractions and components to act against *Chlamydia pneumoniae* infection is presented.

3.1 eNOS activity profiling via systematic fractionation of red wine samples

Initially, the activity guided fractionation of eight selected Austrian red wine samples to isolate eNOS-activating compound(s) was carried out in the “First Test Series” (3.1.1), followed by the “Second Test Series” (3.1.2) and the “Third Test Series” (3.1.3) with the same objective. Chapter 3.1.4 presents the continued fractionation with only one red wine sample in order to isolate and characterize an eNOS-activating compound, and to elucidate the structure using HPLC/UV/MS (3.1.5). Section 3.1.6 illustrates the possible influence of red wine constituents on the generation of H_2O_2 concerning the *in vitro* activation of eNOS. The impact of the relevant red wine component on eNOS mediated release of NO is subject of section 3.1.9. In chapter 3.1.7, eNOS activation of the isolated red wine compound is confirmed by a western blotting experiment. Section 3.1.8 describes the influence on eNOS activity of derivatives of the active red wine constituent. In chapter 3.1.11, information on the improvement of eNOS modulation during vinification is delivered, followed by the eNOS activity measurement of a typical Austrian white wine sample (3.1.12). Furthermore, a quantification of the eNOS activating compound in several wine samples using HPLC is given in chapter 3.1.13. Finally, section 3.1.13 compares the eNOS activation properties of a red wine sample depending on the storage in barrique and steel casks.

3.1.1 The First Test Series

3.1.1.1 Selection of red wine samples

Because the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay cannot be applied as a screening assay with high-throughput capabilities, only a limited number of selected red wine samples were chosen for the eNOS activity profiling.

To systematically approach the identification of eNOS-activating substances in Austrian red wine, 60 representative red wine samples (chapter 2.1.1) were compared regarding their profile of principal components. Eight samples outstanding in polyphenol composition were selected for the First Test Series to measure the eNOS activation of EA.hy926 endothelial cells using the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay. The criteria for the sample selection were very high, respectively very low amounts of the following compounds: catechin, epicatechin, *trans*-resveratrol, *trans*-piceid, total resveratrol content, total anthocyanins and total phenol content.

Red Wine (variety and origin)	Abbre-viation	Catechin	Epicatechin	t-Resveratrol	t-Piceid	Resveratrol (total)	Anthocyanins (total)	Phenols (total)
		mg/L						g/L
Merlot 05 – Carnuntum	MC	93.6	115.2	3.18	2.73	12.7	275	1.82
Zweigelt 05 – Carnuntum	ZC	95.8	128.5	2.63	1.22	6.2	328	1.91
St. Laurent 05 – Klosterneuburg	SK	86.9	57.8	2.40	0.62	5.0	404	1.13
Blaufränkisch 05 – Klosterneuburg	BK	131.3	178.5	2.57	0.40	4.6	239	1.58
Blaufränkisch 05 – Mittelburgenland	BB	109.6	92.5	4.48	0.00	6.9	269	1.34
St. Laurent 04 – Wachau	SW	38.4	31.0	0.13	0.14	0.6	26	0.51
Pinot Noir 04 – Wien	PW	130.9	57.3	1.09	0.22	4.2	41	1.40
Lagrein – 04 Südtirol	LS	27.0	20.0	2.44	2.03	8.1	124	1.51

Table 18: Table of contents of eight red wine samples selected for the First Test Series because of outstanding amounts of relevant components (generated from Table 3). Top contents concerning all 60 red wine samples are marked in green, low contents are marked in red.

3.1.1.2 Fractionation of the red wines

For the first simple fractionation step of the eight selected red wine samples column chromatography was employed using polystyrene beads as stationary phase.

10 % aqueous EtOH (v/v) and 90 % aqueous EtOH (v/v) served as mobile phases (section 2.2.2). The wine samples were not applied directly onto the column, but were tenfold concentrated by rotary evaporation to yield “Dealcoholized Red Wine Concentrates” (DRWCs). These were used for both the fractionation via CC and the direct treatment of EA.hy926 endothelial cells (Figure 4).

Concerning the separation step, the DRWCs were loaded onto the column after equilibration with 10 % aqueous EtOH (v/v), which was used as first eluent to wash out non-aromatic compounds, such as carbohydrates, glycerine and fruit acids. This fraction was collected and named “First Eluate” (FE). Compounds, such as flavonoids, anthocyanins, procyanidins, stilbenes, tannins etc. were retained on the stationary phase and were eluted with 90 % aqueous EtOH (v/v). This fraction was termed “Red Wine Phenol Extract” (RWPE). Both fractions were evaporated and furthermore lyophilized to improve stability. The method is described in detail in the Material and Methods part (chapter 2.2.2). An overview of the first fractionation step is given in Figure 12.

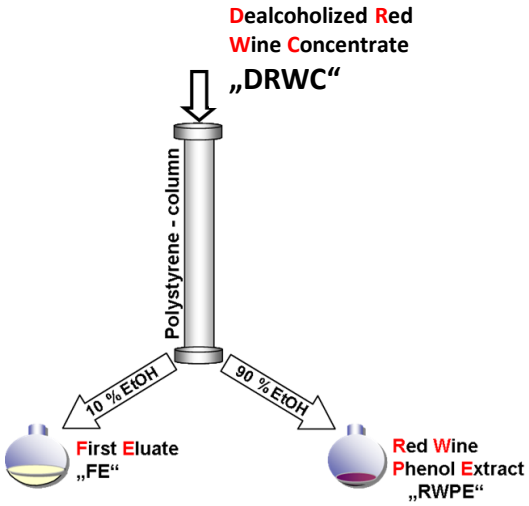


Figure 12: Fractionation scheme to separate the DRWC into the FE and the RWPE by CC using polystyrene.

3.1.1.3 eNOS activity of the fractions of the First Test Series

The consecutive step after the fractionation was the long-term treatment (24 hours) of EA.hy926 cells with the DRWCs and both obtained fractions (FEs and RWPEs).

The initial assays were performed to compare the effect of DRWCs. The concentration of the red wine samples was carried out to avoid any influence of EtOH on treatments. Furthermore, applying small volumina for stimulation prevents dilution of the cell media.

The influence of DRWC on the eNOS after stimulation with 10 µl/ml (according Wallerath et al. [85]) is illustrated in Figure 13. Three DRWCs show a significant enhancement of activity (ZC, MC and BK). The other samples (PW, BB, LS, SK and SW) show the tendency to activate the enzyme.

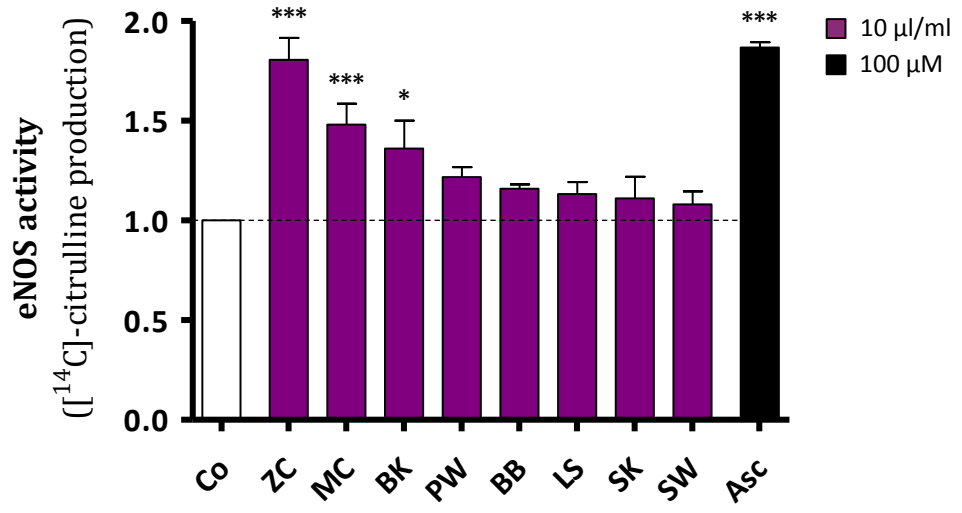


Figure 13: ***"Dealcoholized Red Wine Concentrates" (DRWCs) of the eight selected red wine samples show dissimilar enhancement of eNOS activity.***

EA.hy926 cells were stimulated with the indicated concentration of DRWC for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed. Control cells were treated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) served as positive control. [^{14}C]L-citrulline production was normalized to control (, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) (triplicate, mean ± SEM, n = 3, ANOVA/Bonferroni).*

Then, the FEs were assayed for activation of the eNOS enzyme. Although each of the FEs represents about 90 % of the weight, compared to the dried extracts (chapter 2.2.2) after lyophilisation, they were only tested at 600 µg/ml. This concentration was also used for the RWPEs and allows direct comparison with literature data [71]. The FEs, which should hardly contain phenolic compounds after the separation on polystyrene, did not improve eNOS at all (Figure 14). Hence, the eNOS modulating compound(s) had to be enriched in the RWPEs.

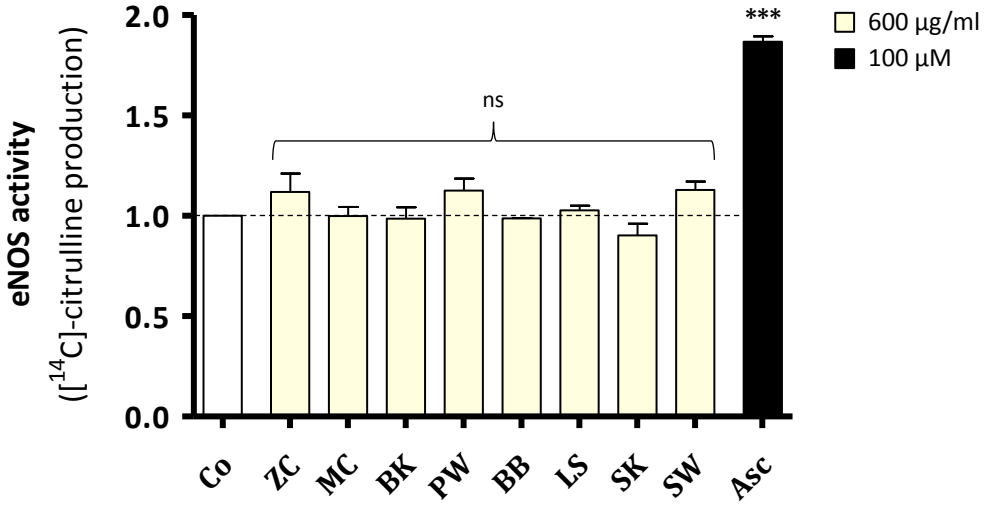


***Figure 14**: "First Eluates" (FEs) do not influence the eNOS activity significantly.*

*EA.hy926 cells were treated with the indicated concentration of FE for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed. Control cells were incubated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) served as positive control. [^{14}C]L-citrulline production was normalized to control (***, $p < 0.001$; ns, not significant) (triplicate, mean ± SEM, n = 3, ANOVA/Bonferroni).*

The RWPEs were screened in two different concentrations (300 µg/ml and 600 µg/ml), and proved the expectation to activate the enzyme, predominantly at the higher concentration. Yet, at the lower concentration (300 µg/ml), differences concerning the eNOS activation could be observed. Five samples (PW, BB, LS, SW and SK) did not alter the activity level, compared to the control, whereas the samples ZC and BK revealed at

least tendencies for an enhancement. MC showed a statistically significant effect concerning the increase of citrulline production, which is associated to enzyme activation.

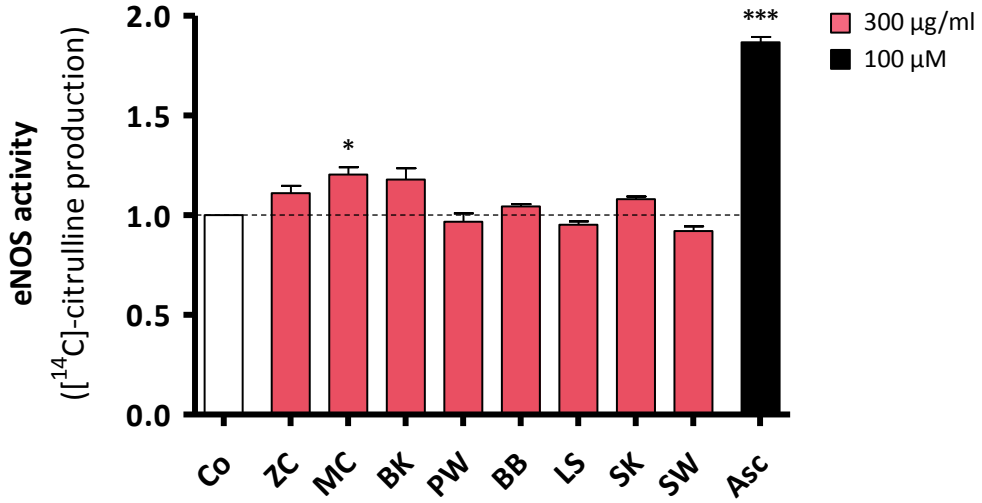


Figure 15: "Red Wine Phenol Extracts" (RWPEs) of only one sample increases the [^{14}C]L-citrulline production at 300 µg/ml.

EA.hy926 cells were incubated with the indicated concentration of RWPE for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was carried out. Control cells were treated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) served as positive control. [^{14}C]L-citrulline production was normalized to control (, $p < 0.05$; ***, $p < 0.001$) (triplicate, error bars = mean ± SEM, n = 3, ANOVA/Bonferroni).*

Complementary to the results of the FEs at 600 µg/ml and the RWPEs at 300 µg/ml, the RWPEs of the eight samples were investigated for their effect on eNOS activity at 600 µg/ml. The previous Figure 14 and Figure 15 illustrated no effect or only a slight effect; the treatment of EA.hy926 cells with RWPEs of the eight Austrian red wine samples at 600 µg/ml confirmed, that eNOS-activating (phenolic) compounds of red wine were enriched to an adequate concentration in RWPEs. Five samples showed an enhancing effect on the activity (ZC, MC, BK, PW, SK), and three RWPEs did not influence the eNOS significantly as shown in Figure 16. These are BB, LS and SW.

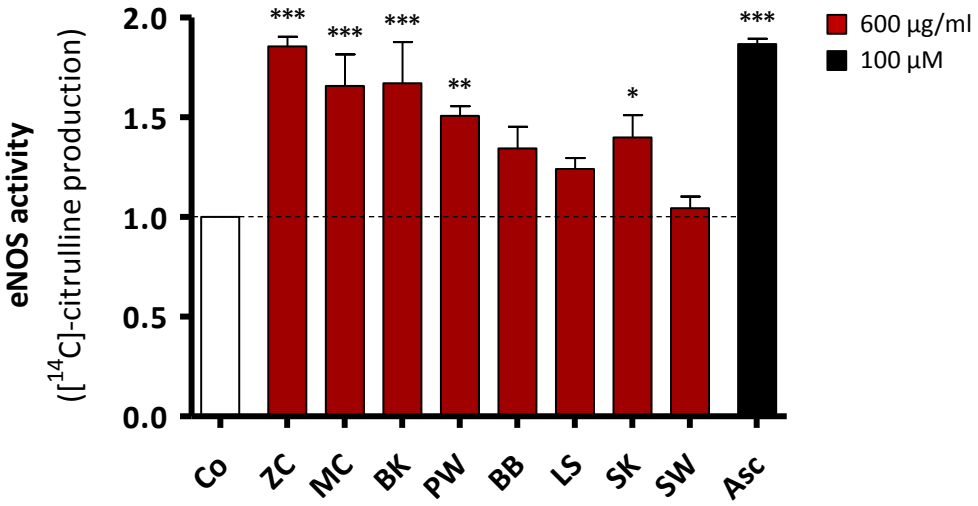


Figure 16: "Red Wine Phenol Extracts" (RWPEs) of five red wine samples demonstrate an enhancing effect on eNOS activity, tested at 600 µg/ml.

EA.hy926 cells were treated with the indicated concentration of RWPE for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed. Control cells were stimulated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) was used as positive control. [^{14}C]L-citrulline production was normalized to control (, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) (triplicate, mean ± SEM, n = 3, ANOVA/Bonferroni).*

Regarding the First Test Series, it has to be pointed out that concentrations used for the eNOS activity testing are related to literature [69-71]. The effect after stimulation with 10 µl/ml of DRWC varied depending on the red wine sample. The same holds true for the RWPEs, whose activity on eNOS was found to be concentration dependent.

The polyphenol content of 300 µg/ml of the RWPEs used for stimulation of endothelial cells was comparable to the amount contained in 10 µl/ml of the DRWCs.

Unfortunately, those two treatments illustrated a noticeable difference of the eNOS activity fingerprint (see Figure 13 and Figure 15), suggesting that the reduced effect at 300 µg/ml is related to the fractionation step of DRWC into FE and RWPE using CC. Possible reasons for that could be loss of eNOS-activating compound(s) on the stationary phase polystyrene due to incomplete elution with 90 % EtOH (v/v), or a synergistic effect of FE and RWPE constituents.

The RWPEs were also tested at another concentration (600 µg/ml), which yielded higher eNOS activity for several red wine samples (see Figure 14). Thus, this concentration appeared to contain effective amounts of the appropriate polyphenol(s).

3.1.2 Second Test Series

The criterion for the sample selection to proceed with a Second Test Series was to choose four RWPEs with the most promising results in the First test series. These were the Zweigelt – Carnuntum (ZC), the Merlot – Carnuntum (MC), the Blaufränkisch – Klosterneuburg (BK) and the Pinot Noir – Wien (PW). These four Austrian red wine samples´ RWPEs were further fractionated and tested as described below.

3.1.2.1 Fractionation of the Red Wine Phenol Extracts

The RWPEs of the four samples selected for the Second Test Series, which were stored at -20 °C as lyophilized powders to sustain stability and activity, were dissolved in the smallest possible amount of 70 % aqueous EtOH (v/v). Then, each sample was diluted with distilled water (400 ml/g RWPE), transferred into a separation funnel, and a liquid-liquid partition using ethyl acetate (200 ml/g RWPE) as organic phase was performed. After repeating the partition consecutively three times with fresh ethyl acetate, the resulting fractions were labeled as “Apolar Fraction” (AF; ethyl acetate) and “Polar Fraction” (PF; H_2O). They were evaporated, redissolved again, and finally lyophilized and stored at -20 °C. The dried AF of each sample was one third of the weight compared to the related RWPE, while each PF represented the complementary 67 %, on average. Figure 17 illustrates the fractionation scheme of the Second Test Series.

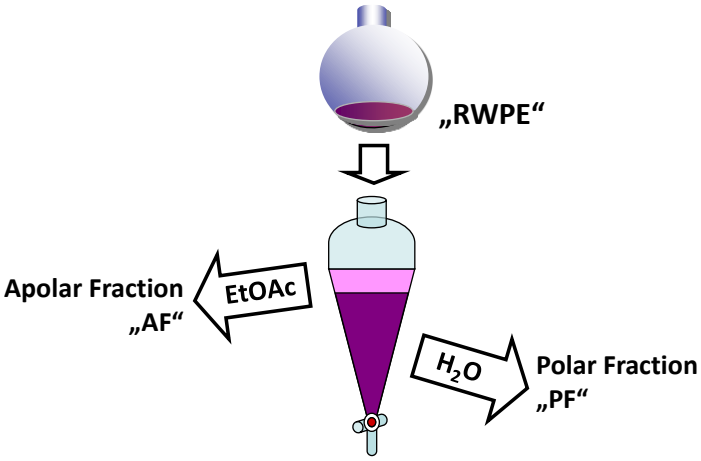


Figure 17: Fractionation of RWPE into AF and PF by liquid-liquid partition using H_2O and ethyl acetate.

3.1.2.2 Influence of the Apolar Fraction and the Polar Fraction on eNOS activity

The [^{14}C]L-arginine/[^{14}C]L-citrulline conversion experiments of the Second Test Series were modified according to the obtained amounts of the AFs and the PFs. The weight was related to their initial fractions, the RWPEs. As mentioned in chapter 3.1.2.1 of the Second Test Series, the ratio of the yielded amounts of AF to PF was about 1:2.

Within the First Test Series the RWPEs were assayed at 600 µg/ml, revealing a high induction of eNOS activity. For the current test series an alternative approach was performed, whereby the endothelial cells were stimulated with AFs at a concentration of 200 µg/ml and with PFs at 400 µg/ml, corresponding to 600 µg/ml RWPE. In addition to the experimental comparison of AF and PF with RWPE, the AF was recombined with the PF (AF + PF) and matched directly with the RWPE to have a stability and solubility control for the fractions, because previous studies showed a reduction of activity when performing a similar experiment [69].

The *in vitro* result, shown in Figure 18, demonstrates an effect of the AFs at 200 µg/ml on the eNOS enzyme. It is completely reproducible in relation to the RWPE and the recombined AF + PF, both measured at 600 µg/ml. For three out of the four samples (BK, the MC, and the ZC) the activity pattern was identical. PW was the only sample, where PF influenced the enzyme activity compared to its AF.

To conclude, the eNOS activating compounds are predominantly present in the AFs.

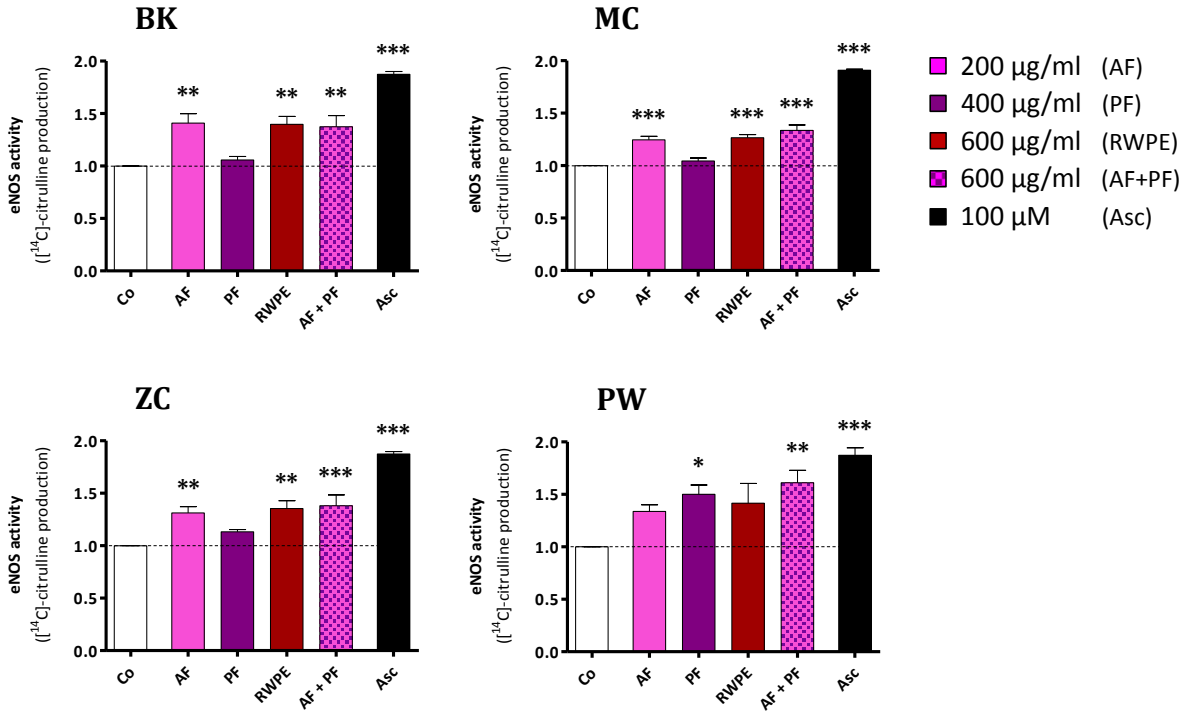


Figure 18: For three out of the four samples, the eNOS activating compound(s) are particularly enriched in the AF

EA.hy926 cells were incubated with the indicated concentrations for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed. Control cells were stimulated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) served as positive control. [^{14}C]L-citrulline production was normalized to control (, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) (triplicate or quadruplicate, mean ± SEM, n = 3 - 4, ANOVA/Bonferroni).*

3.1.2.3 HPLC of the eNOS-activating Apolar Fractions

Based on the results of the biological testing of the Second Test Series (section 3.1.2.2) the HPLC-fingerprints of the four AFs were compared using an established HPLC-method (method A1; section 2.3.1). The idea was to analyze the pattern of the eNOS-activating AFs by HPLC, determining quantitative and qualitative dissimilarities in the shown chromatograms. The detection was performed at 280 nm (UV), a universal wavelength to analyze phenols, together with an evaporative light scattering detector (ELSD), which is a universal detector allowing estimation of the relative quantities of detected compounds within the chromatogram of each fraction. Further details of the

HPLC-system are mentioned in section 2.3.1. Generally, it can be concluded that no qualitative differences could be detected in the peak pattern, neither at 280 nm nor with ELSD. However, the variation of the fingerprints of the AFs refers to the quantitative deviation of single compounds. Worth mentioning are the peaks with retention times at 13, 45, 51, 62, 68, 78, 80, 88 and 92 minutes (see Figure 19 and Figure 20 below).

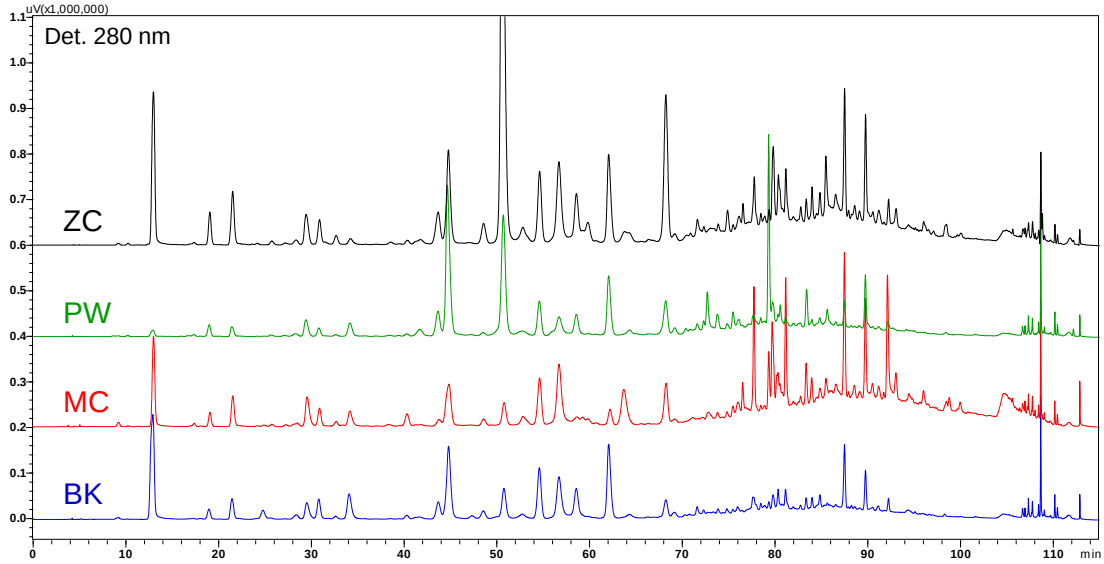


Figure 19: HPLC fingerprints of the four samples´ AFs of the Second Test Series, detected at 280 nm (method A1; 2.3.1).

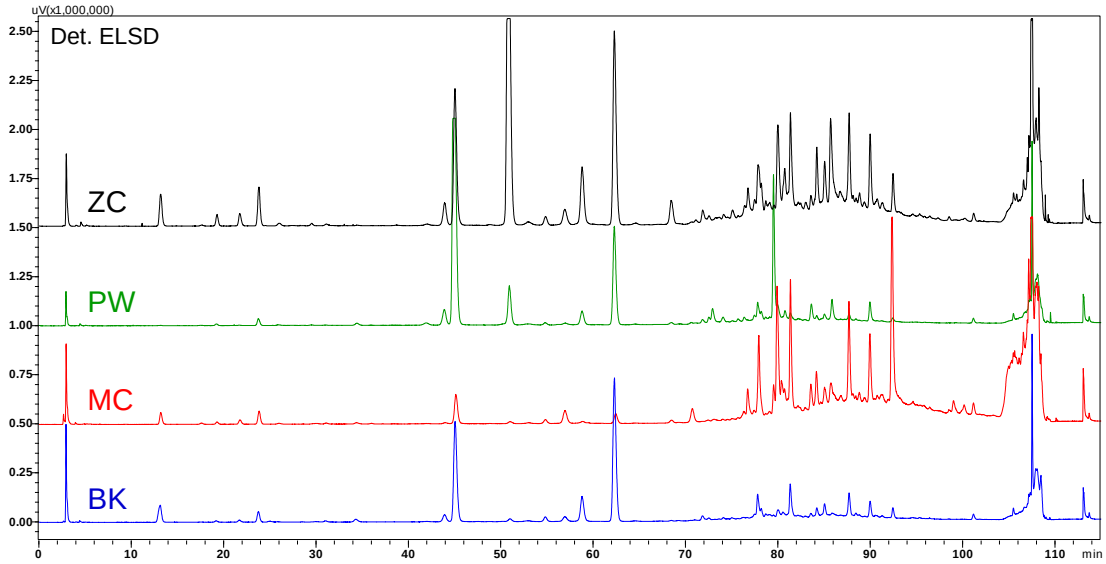


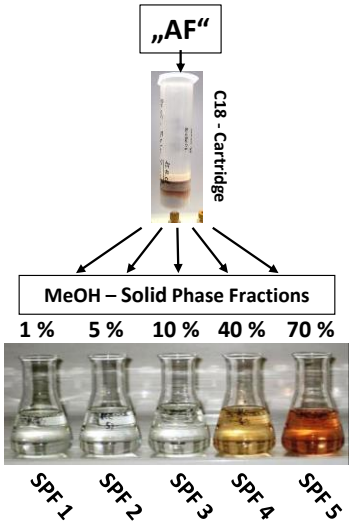
Figure 20: HPLC fingerprints of the four samples´ AFs of the Second Test Series detected with ELSD (method A1; 2.3.1).

3.1.3 Third Test Series

Based on the results of the Second Test Series only one sample was chosen to continue the eNOS activity guided isolation for relevant compound(s). The sample for the Third Test Series was the Blaufränkisch – Klosterneuburg (BK), more precisely the AF of this red wine. This sample was selected due to the strongest activation of eNOS compared to the other AFs (see Figure 18).

3.1.3.1 Fractionation of the Apolar Fraction

For the preparation of the fractions to use for the Third Test Series, AF of BK (200 mg) was solved in approximately 300 µl of 70 % EtOH (v/v) and loaded onto the equilibrated C18-cartridge for SPE (chapter 2.2.4). Then, a stepwise MeOH gradient (1 %, 5 %, 10 %, 40 % and 70 %) was used to elute fractions from the stationary phase. They were named as "Solid Phase Fraction" (SPF) 1, 2, 3, 4 and 5, respectively. Then, the SPFs were concentrated by rotary evaporation and dried in plastic tubes (2 ml) using a SpeedVac. Finally, their relative weight compared to the superior AF was determined to stimulate endothelial cells related to their relative percentage (depicted in Figure 21).



Fraction	Conc. (rel. to AF)	% (rel. to AF)
SPF 1	42 µg/ml	21 %
SPF 2	28 µg/ml	14 %
SPF 3	27 µg/ml	13.5 %
SPF 4	84 µg/ml	42 %
SPF 5	19 µg/ml	9.5 %
AF	200 µg/ml	

Figure 21: Scheme of the "Solid Phase Fractions" (SPF) 1 – 5 obtained by SPE, and their relative quantities compared to the initial AF.

3.1.3.2 Influence of the Solid Phase Fractions on eNOS activity

The influence of the "Solid Phase Fractions" (SPFs) 1 – 5 of BK on the eNOS was measured with the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay analogously to the Second Test Series. In detail, the SPFs were tested relatively to the concentration of AF (200 µg/ml equals 100 %) (see Figure 21 and Figure 22). The endothelial cells were stimulated with 42 µg/ml of SPF 1 (21 %), 28 µg/ml of SPF 2 (28 %), 27 µg/ml of SPF 3 (13.5 %), 84 µg/ml of SPF 4 (42 %), and 19 µg/ml of SPF 5 (9.5 %).

The results of SPF 1 – 5 compared to their AF indicated that SPF 1, SPF 2 and SPF 5 did not modulate the eNOS activity level at all. The only SPF to enhance the activity significantly and similar to the AF was SPF 3, tested at 27 µg/ml (13.5 %). SPF 4 showed a tendency to activate eNOS, but this effect was not significant at the tested concentration (Figure 22).

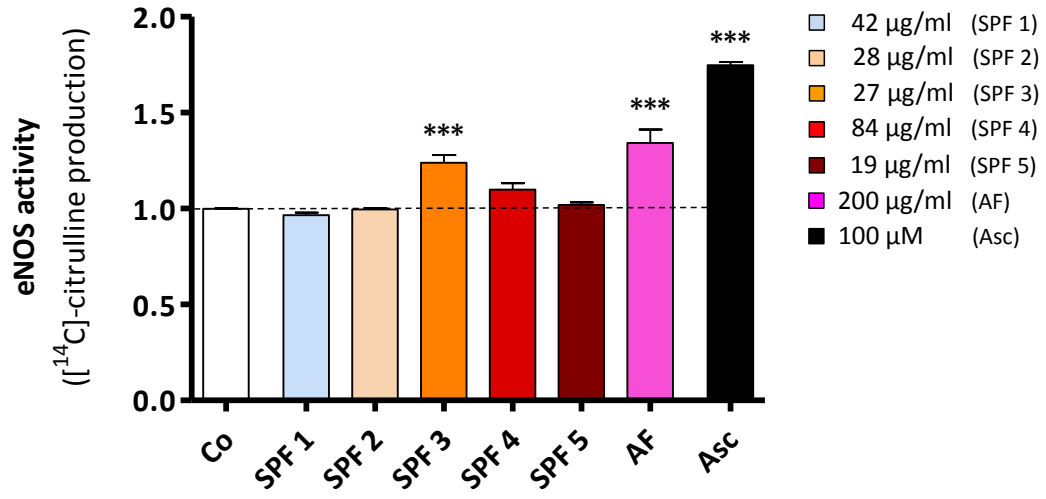


Figure 22: "Solid Phase Fractions" (SPF) 1 – 5 of BK sample were tested together with AF for eNOS activation, and SPF 3 represents an effect.

*EA.hy926 cells were incubated with the indicated concentrations for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed. Control cells were stimulated with DMSO (0.1 %); ascorbic acid (Asc, 100 µM) served as positive control. [^{14}C]L-citrulline production was normalized to control (***, $p < 0.001$) (quadruplicate, mean ± SEM, $n = 4$, ANOVA/Bonferroni).*

3.1.3.3 HPLC of the Solid Phase Fractions and the Apolar Fraction of the Blaufränkisch Klosterneuburg (BK) red wine sample

The comparison of the HPLC-chromatograms was employed with analytical method A1 (see section 2.3.1). The chromatograms of SPF 1 – 5 as well as AF, detected at 280 nm, are shown in Figure 23. The single chromatograms revealed differences in their fingerprints. SPF 1 contains mainly compounds belonging to the group of phenolcarbonic acids, SPF 2 and SPF 3 are primarily cinnamic acid derivatives and the fractions SPF 4 and SPF 5 are composed of flavonoids, anthocyanins and stilbenes.

Since the eNOS activity measurement of SPF 1 – 5 demonstrate that mainly SPF 3 and maybe to a lower extent SPF4 showed an effect, only compounds which were detected in these subfractions could be mainly responsible for influencing the eNOS. Consequently, HPLC-fingerprints of the subfractions were compared, and components of inactive fractions, such as SPF 1, SPF 2, and SPF 5 were considered inactive. SPF 4 showed at least tendency to increase enzyme activity, which could be effected either by a lower quantity of SPF 3´s activator or by another compound, contained in SPF 4.

As a consequence of comparing the chromatograms at the active and inactive fractions, the most obvious signal in the most active SPF 3 is detected at 56 minutes, showing a UV spectrum very similar to gallic acid. However, the stronger retention of this compound on the modified C18 column compared to gallic acid (peak at 13 minutes), indicated possibly a gallic acid derivative with a lipophilic substituent, which is not influencing the chromophore.

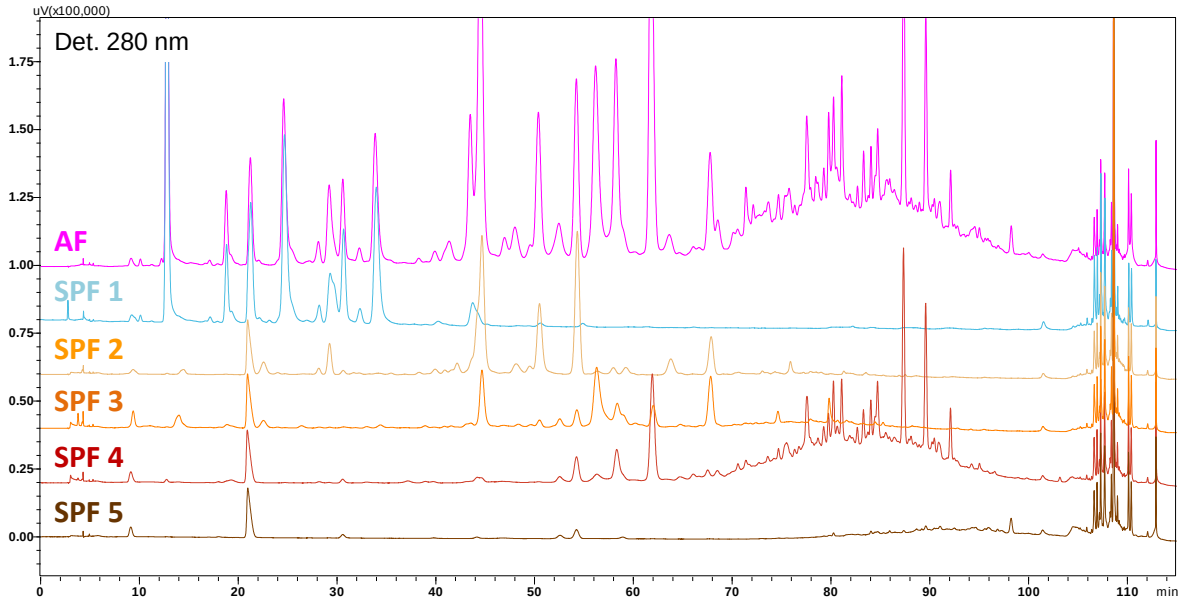


Figure 23: HPLC-chromatograms of the subfractions SPF 1 – 5 of AF (BK) of the third test series detected at 280 nm (method A1; 2.3.1).

To confirm the above mentioned assumptions, further fractionation of SPF 3 in a large scale would have been necessary. Unfortunately, neither the red wine sample Blaufränkisch Klosterneuburg 2005 nor fractions or subfractions were left in an adequate amount for isolation of reasonable amounts for further biological testing and for structure elucidation via NMR.

3.1.4 Continued eNOS activity guided fractionation

3.1.4.1 Restart of the fractionation with the sample Burgweingartl 2007 (BWG 07)

Since large amounts of BK 05 were not available, two new red wine samples were provided by the cooperators from Klosterneuburg instead. These were two Cuveés with the same appellation, containing mainly Blaufränkisch. The wine was called "Burgweingartl" (BWG) and vintages of 2007 and 2008 were provided.

To choose the most active sample, these red wines – BWG 07 and BWG 08 – were compared with the initial BK 05 using the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay. Thus, the three pure red wines were tested at two concentrations, 50 µl/ml and 100 µl/ml. The aim of this comparison was to find out, if the BWG samples show the

ability to enhance the enzyme activity to a similar level as BK 05. The results are pictured in Figure 24.

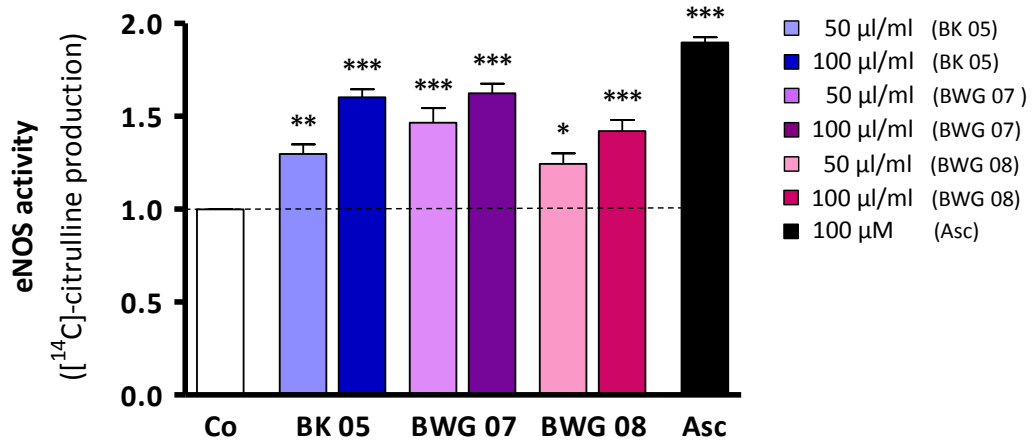


Figure 24: BWG 07 and BWG 08 were compared to BK 05 for their ability to increase eNOS activity.

EA.hy926 cells were stimulated with the indicated concentrations for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed. Control cells were treated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) was the positive control. [^{14}C]L-citrulline production was normalized to control (, p < 0.05; **, p < 0.01; ***, p < 0.001) (quadruplicate, mean ± SEM, n = 3, ANOVA/Bonferroni).*

Both red wines, BWG 07 and BWG 08, were generally able to influence the enzyme positively, but only BWG 07 showed a level comparable to BK 05. Thus, the fractionation was restarted with BWG 07 in the identical way already described in chapters 3.1.1.2 and 3.1.2.1

First, the red wine was concentrated and divided by column chromatography into the FE and the RWPE. Second, the RWPE was separated into the AF and the PF by liquid-liquid partition between water and ethyl acetate. Then, the effect of these four fractions on [^{14}C]L-citrulline production was measured and compared to the previous results (chapter 3.1.2.2) for the fractions of BK 05 (Figure 25). The activity pattern of BWG 07 was expected to resemble that one of BK 05, where the eNOS activation was mostly attributed to the AF. However, the result of BWG 07 differed from BK 05, because its activity was distributed to both, the AF and the PF. However, in the

previously performed test series all AFs were proven to effect eNOS (see Second Test Series chapter 3.1.2.2). Thus, the continued fractionation focused on the BWG 07´s AF.

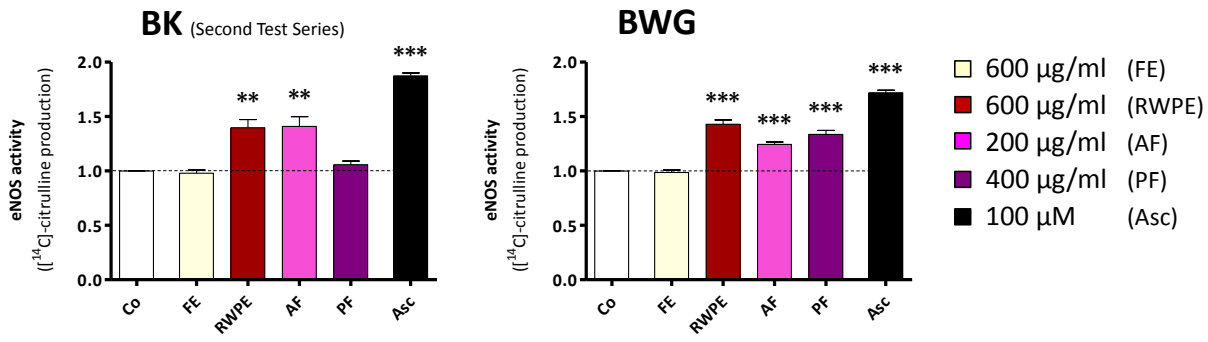


Figure 25: eNOS activity patterns of fractions of BK 05 and BWG 07.

*EA.hy926 cells were treated with the listed concentrations for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was carried out. Control cells were incubated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) served as positive control. [^{14}C]L-citrulline production was normalized to control (**, p < 0.01; ***, p < 0.001) (quadruplicate, mean ± SEM, n = 3 - 4, ANOVA/Bonferroni).*

3.1.4.2 Semipreparative HPLC-fractionation of the Apolar Fraction of Burgweingartl 07

After the repetition of the first two fractionation steps, known from previous experiments (chapter 3.1.1.2 and 3.1.2.1), the method for the isolation of the active compound(s) was changed from the SPE (section 3.1.3.1) to a semipreparative HPLC-system using a timed-cut method to collect defined fractions. The upscaling from an analytical HPLC-system to a semipreparative HPLC-system was associated with a change of stationary phase and also a gradient transfer. Usually, in case of performing an HPLC-upscaling it would be ideal to utilize a stationary phase with the same chemistry, but the analytical column (Aquasil® C18) was not available in semipreparative dimensions. Thus, a semipreparative Purospher® C18e column was applied for this continued fractionation. Acetonitrile (see HPLC-method A1 in section 2.3.1) as one part of the mobile phase ought to be replaced by MeOH to reduce

operating costs and toxicity, although this is accompanied with a change of selectivity, expressed as modified retention of the compounds.

To run the Purospher® C18e semipreparative column (250x10 mm, 5 µm) some parameters of the analytical method (method A1 in section 2.3.1) had to be optimized. The flow is generally influencing the efficiency of separation. Therefore, the flow was adapted in relation to the diameter of 10 mm, and was set at 4.7 ml/min. The correlating backpressure in the HPLC had to be controlled during the setup to keep it below 300 bar, in order to prevent equipment and column from damage. The temperature, which is a very important parameter for the quality of separation, was adjusted to 30 °C, resulting in a maximum pressure of about 270 bar.

Figure 26 represents the chromatogram using the semipreparative method. In line with the former fractionation procedure and the HPLC-characterization of the Third Test Series (chapter 3.1.3.3) the separation was focused on the more polar components of the AF of BWG 07, particularly on those eluting between 40 and 65 minutes.

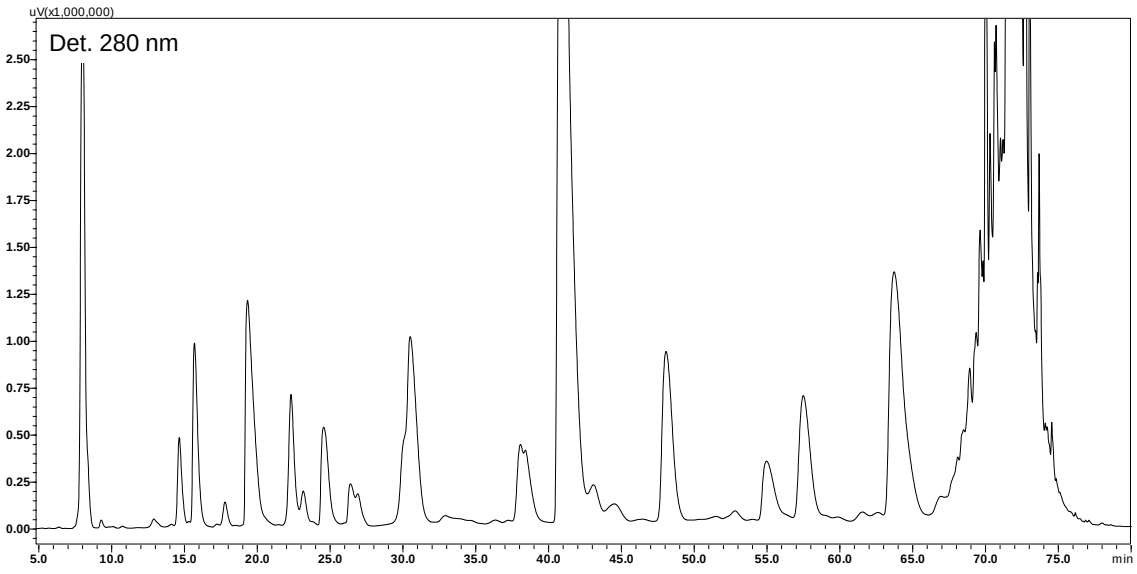


Figure 26: Representative HPLC-chromatogram of the semipreparative fractionation, detected at 280 nm (method P1; chapter 2.2.5).

The obtained subfractions of AF were tested in the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay. As 6-well plates provide four wells for the treatment of endothelial cells with samples (besides control and positive control), four semipreparative HPLC-fractions were obtained, of which the active one was further fractionated two more times (Figure 27, Figure 29, Figure 31).

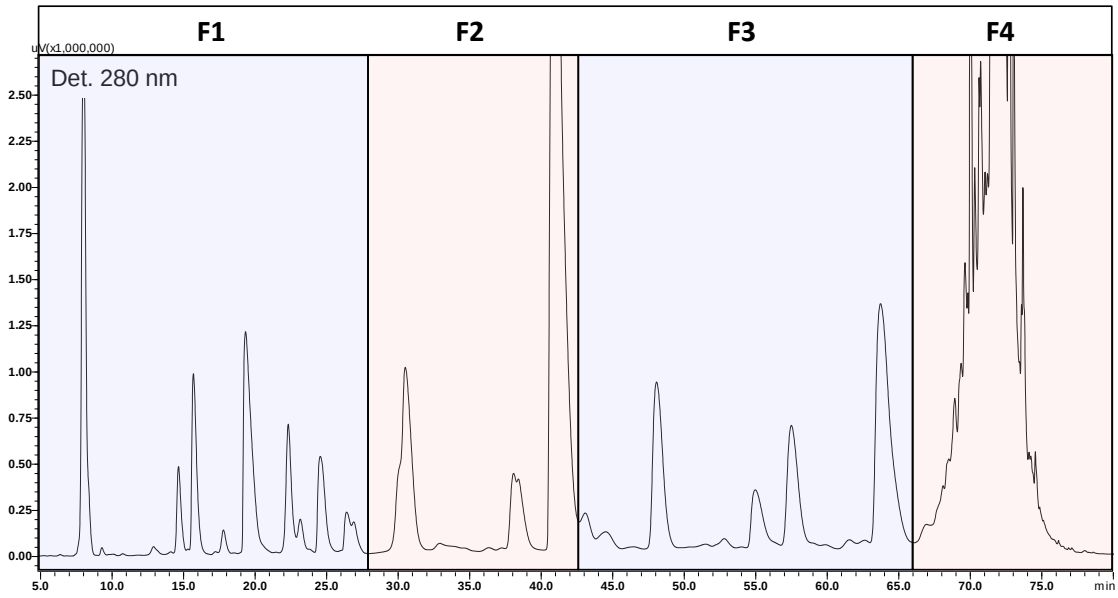


Figure 27: Chromatogram of the first semipreparative fractionation applying the timed cut (27.9 min; 42.6 min; 66.0 min and 79.5 min) to achieve four fractions, detected at 280 nm (method P1; chapter 2.2.5).

The assaying of the obtained fractions showed that F1 and F2 had no effect on the eNOS, in contrast to the active fractions F3 and F4 (see Figure 28). The stimulation of the endothelial cells with F3 caused a higher activation than with F4. The fractionation was only continued with F3 for two reasons. F3 showed better eNOS activation compared to F4 and contained a considerably lower number of compounds, which could be responsible for the eNOS activity enhancement.

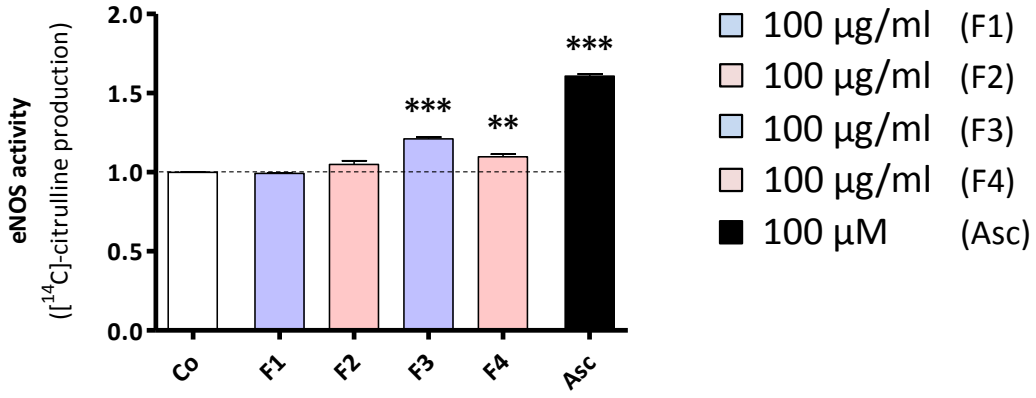


Figure 28: The fractions F1 – F4 partially alter the [^{14}C]L-citrulline production. F3 and F4 have an effect on eNOS activation.

*EA.hy926 cells were incubated with the shown concentrations for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed. Control cells were stimulated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) served as the positive control. [^{14}C]L-citrulline production was normalized to control (**, p < 0.01; ***, p < 0.001) (triplicate, mean ± SEM, n = 3, ANOVA/Bonferroni).*

The fractionation of F3 yielded the subfractions F3.1, F3.2, F3.3 and F3.4. The time range of the relevant section started at 42.6 minutes and ended at 66.0 minutes in the chromatogram, and fractions were further tested for eNOS activation.

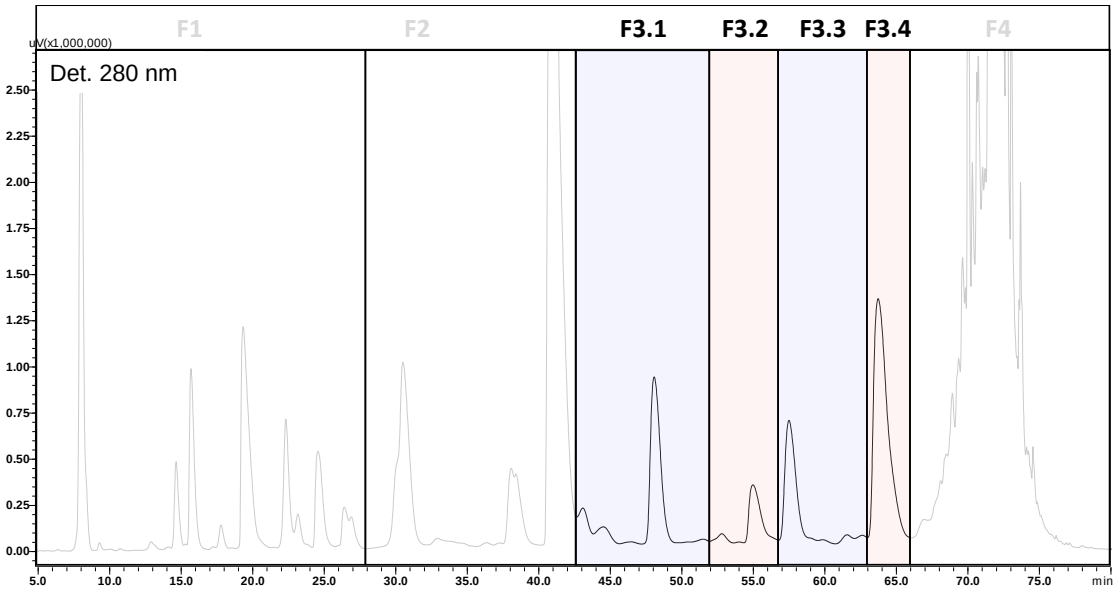


Figure 29: Fractions F3.1 – F3.4 of the AF of BWG 07, obtained by semipreparative HPLC, at 280 nm (method P1; chapter 2.2.5).

The single subfractions, illustrated in Figure 29, were obtained by the semipreparative HPLC-fractionation of F3 in almost identical amounts. Therefore, they were assayed at the same concentration (25 µg/ml) and compared to the initial F3 at 100 µg/ml. The eNOS activating effect of F3 was fully recovered in subfraction F3.3, whereas the other subfractions F3.1, F3.2 and F3.4 remained at the control level.

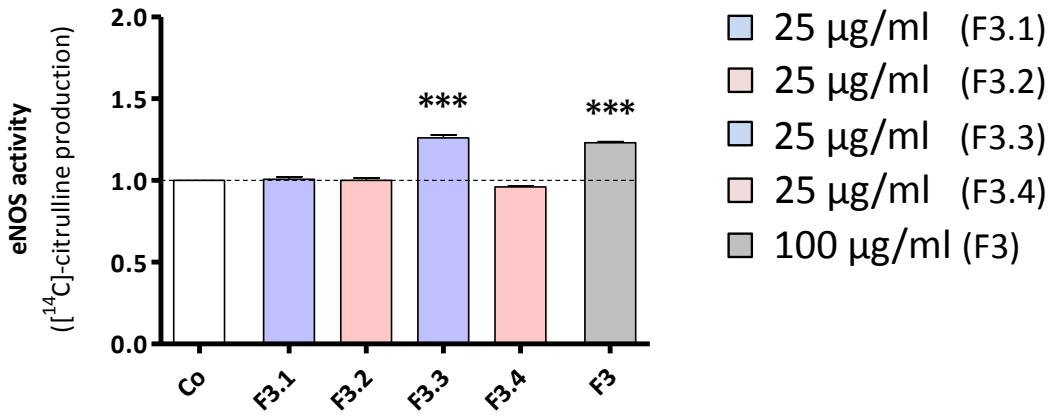


Figure 30: The subfractions F3.1 – F3.4 were tested together with their superior fraction F3, and only F3.3 had influence on eNOS activation.

*EA.hy926 cells were stimulated with the listed concentrations for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed. Control cells were treated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) was used as positive control. [^{14}C]L-citrulline production was normalized to control (***, $p < 0.001$) (triplicate, mean ± SEM, n = 3, ANOVA/Bonferroni).*

Finally, the fractionation was refined a third time with F3.3 (from 56.8 minutes to 63.1 minutes in the chromatogram; see Figure 31), and again four fractions were gathered. The enlarged section of the chromatogram illustrates the relevant peaks (see Figure 31), representing F3.3.1, F3.3.2, F3.3.3, and F3.3.4. The four subfractions were tested for eNOS activation at concentrations according to their relative weight and compared to F3.3 at 25 µg/ml (Figure 32).

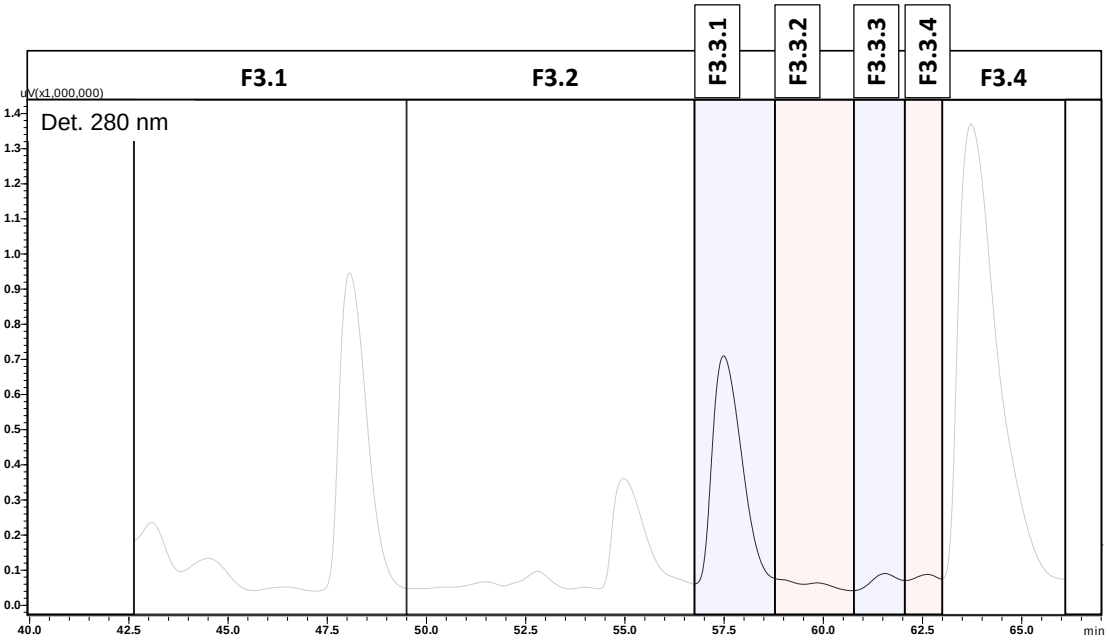


Figure 31: Enlarged section of the chromatogram showing possible eNOS-activating subfractions of the AF of BWG 07, detected at 280 nm (method P1; chapter 2.2.5).

The eNOS activity guided fractionation of BWG 07´s AF resulted in the isolation of a single compound, represented as fraction F3.3.1, which seems to be solely responsible for the measured eNOS activation by F3.

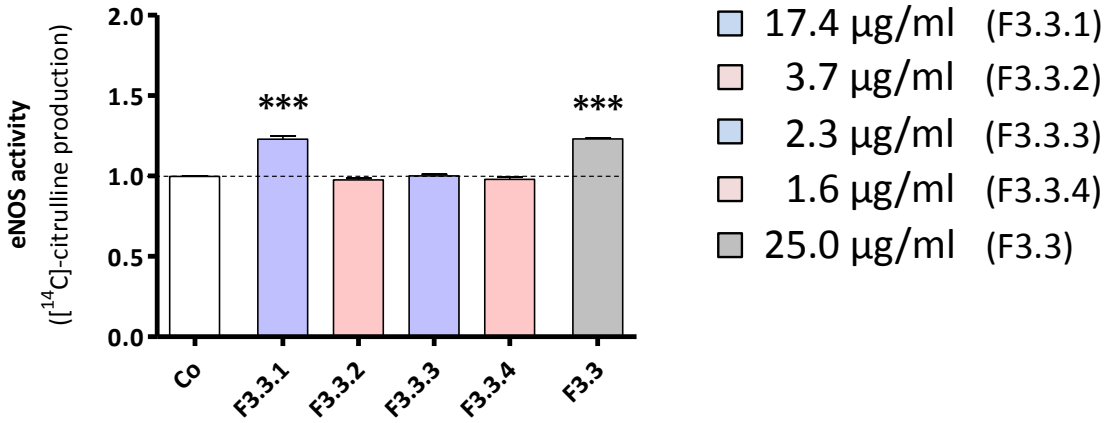


Figure 32: Fraction F3.3.1 enhances the enzyme activity comparable to F3.3.

*EA.hy926 cells were incubated with the concentrations for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was carried out. Control cells were stimulated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) was positive control. [^{14}C]L-citrulline production was normalized to control (***, $p < 0.001$) (triplicate, mean ± SEM, n = 3, ANOVA/Bonferroni).*

The performed bioassay guided fractionation turned out to be successful. Starting from the wine samples and using adequate separation techniques like CC, liquid-liquid partition, SPE, and repeated semipreparative HPLC, one compound showing significantly increased eNOS activity was obtained. The complete fractionation scheme is illustrated below.

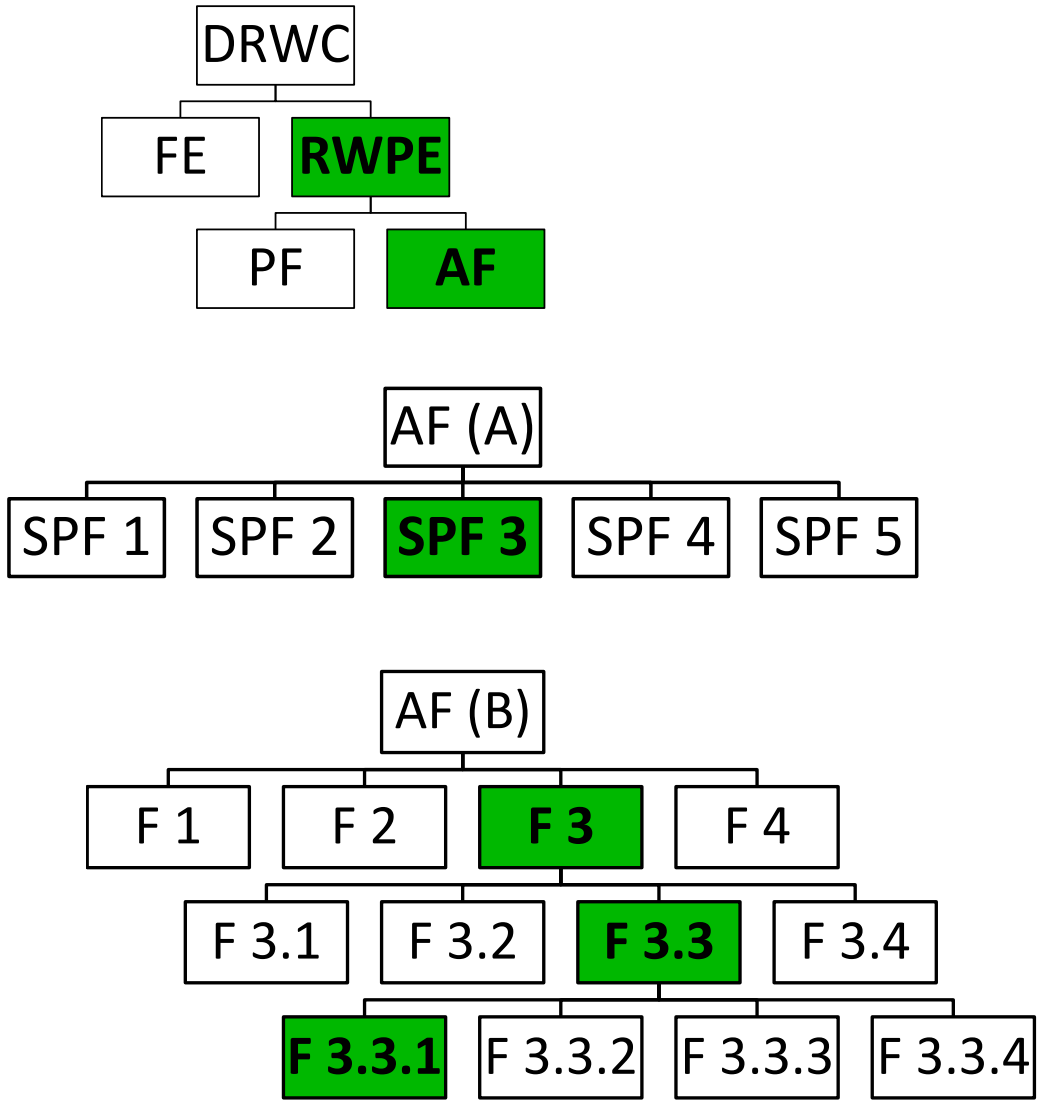


Figure 33: Scheme of the eNOS activity guided fractionation of red wine samples to isolate active compounds. The active fractions are marked green. Consecutive fractionation of AF is depicted in two different ways (A, B).

A detailed characterization to identify this eNOS-activator as well as further investigations are described in the following chapters.

3.1.5 Characterization of the isolated eNOS-activating red wine compound and two related structures using HPLC-DAD/MS

The identification of the isolated red wine compound (see chapter 3.1.4.2) was based on the UV-spectra, which were recorded with a diode array detector (DAD), and mass spectrometry (MS), coupled to HPLC. The proposed structure was finally confirmed by comparison of the characteristics to reference substances.

The HPLC-chromatogram is pictured in Figure 34 and HPLC-method A3 is described in the materials and methods part (see chapter 2.3.2). This method was extended compared to the existing analytical HPLC-method A1 (see chapter 2.3.1), to enable a better HPLC/MS-characterization of the peaks.

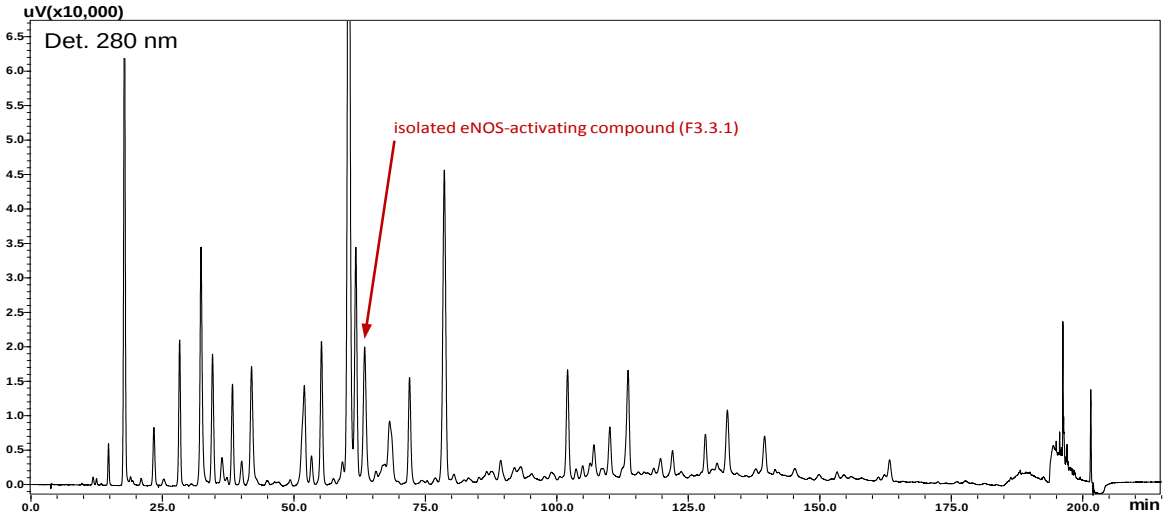


Figure 34: Chromatogram of the AF of BWG 07 at 280 nm, using the extended method A3 (chapter 2.3.2) for LC/MS analysis.

The eNOS-activating compound, represented by fraction F3.3.1 (Figure 31), has a retention time of 63.7 minutes in the used HPLC-system. The UV-spectrum shows two maxima at 273 nm and at 217 nm, which are quite typical for gallic acid and its derivatives. However, the retention time as well as the MS^n spectra differed from gallic acid as reference compound. The mass spectrometry measurement (ESI) was carried out in the negative ion mode and a quasi-molecular ion of m/z 196.7 was detected, indicating a MW of 198 Da. The MS^2 spectrum shown in Figure 35 indicated the neutral loss of an ethylene group (-28), yielding a fragment ion at m/z 168.7, typical for gallic acid, and a further loss of CO_2 related to its carboxyl group.

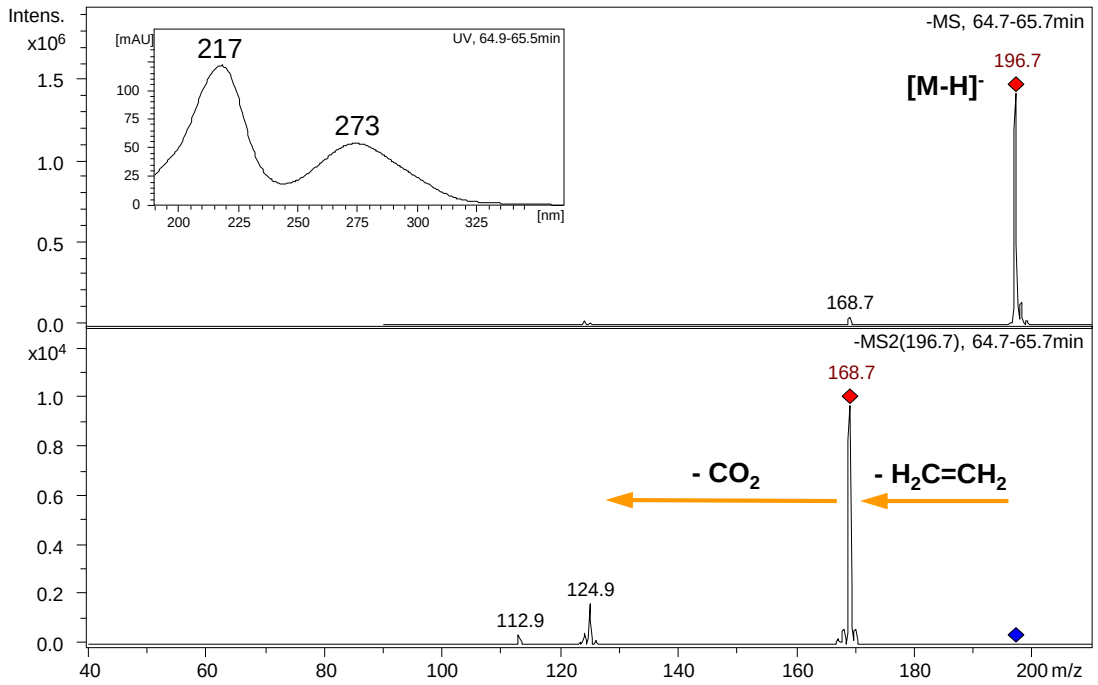


Figure 35: UV spectrum and MS-fragmentation pattern of the isolated compound ethyl gallate

Thus, the most probable suggestion for the structure of this compound was the ethyl ester of gallic acid, also termed as ethyl gallate (EG). To confirm this assumption, ethyl gallate was purchased as reference compound. It showed the same properties as the isolated compound from F3.3.1 with regard to retention time, UV spectrum and MS-fragmentation pattern.

Therefore, the isolated compound, showing significant eNOS activity, could unequivocally be identified as ethyl gallate (gallic acid ethyl ester)(Figure 36).

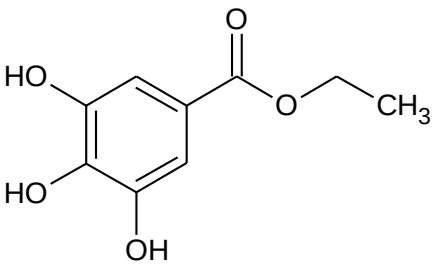


Figure 36: Structure of the identified isolated compound ethyl gallate (gallic acid ethyl ester).

After the structure elucidation a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed with the commercial sample to prove the influence of EG on eNOS activation. The ethyl gallate content in red wine was compared to literature[4], and the pure substance was tested at three different concentrations (see Figure 37). The gallic acid ethyl ester possessed an enhancing effect on the eNOS activity after long-term treatment with 10 µM, 50 µM and 100 µM, but without a clear dose-dependency. The obtained level of eNOS activity was the same as the AF and its consecutively obtained activating fractions of the BWG 07, respectively (see chapter 3.1.4.2). The highest eNOS activity can be observed at the 50 µM concentration

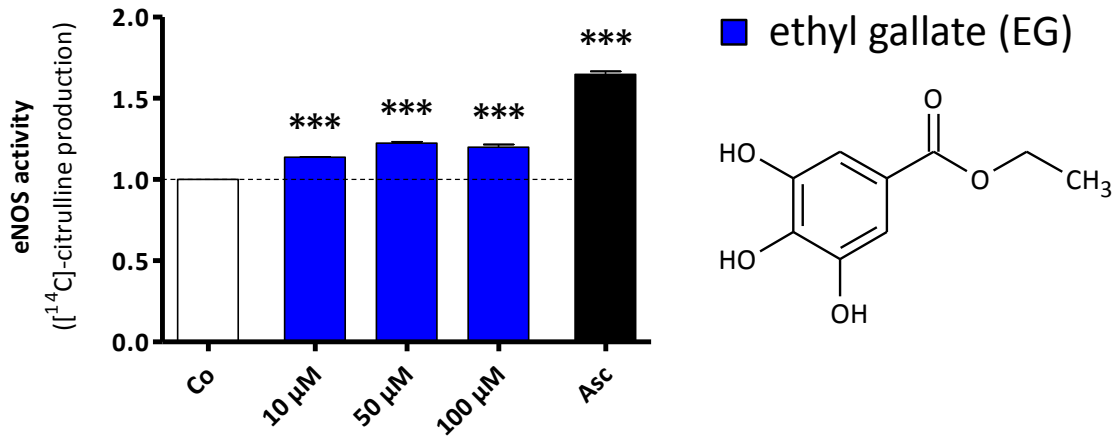


Figure 37: The identified red wine compound ethyl gallate (gallic acid ethyl ester) increases the enzym activity significantly at the tested concentrations.

*EA.hy926 cells were treated with the indicated concentrations for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed. Control cells were stimulated with DMSO (0.1 %), using ascorbic acid (Asc, 100 µM) as positive control. [^{14}C]L-citrulline production was normalized to control (***, $p < 0.001$) (triplicate, mean ± SEM, n = 3, ANOVA/Bonferroni).*

In addition to EG, free gallic acid (GA) could also be identified in the AF of the red wine sample BWG 07.

Gallic acid has the same UV spectrum as its derivative ethyl gallate with maxima at 273 nm and 216 nm and therefore, the two compounds could not be distinguished by

[4] http://www.phenol-explorer.eu/ access: 2011/08/08

their photometric properties. The first important attribute to differentiate between the two compounds is the retention time, which varies remarkably because of the changed polarity related to the substituted ethyl group. GA has a retention time of about 17 minutes in the applied HPLC-method A3 (chapter 2.3.2), while EG elutes at about 65 minutes using this method. The mass spectrometry analysis revealed data concerning the fragmentation of GA in the negative ESI-mode. The MS showed a quasi-molecular ion at m/z 168.7 confirming the MW of 170 of gallic acid similar to the MS^2 of ethyl gallate (see Figure 35 above), and another signal at m/z 124.9 explaining the CO_2 loss.

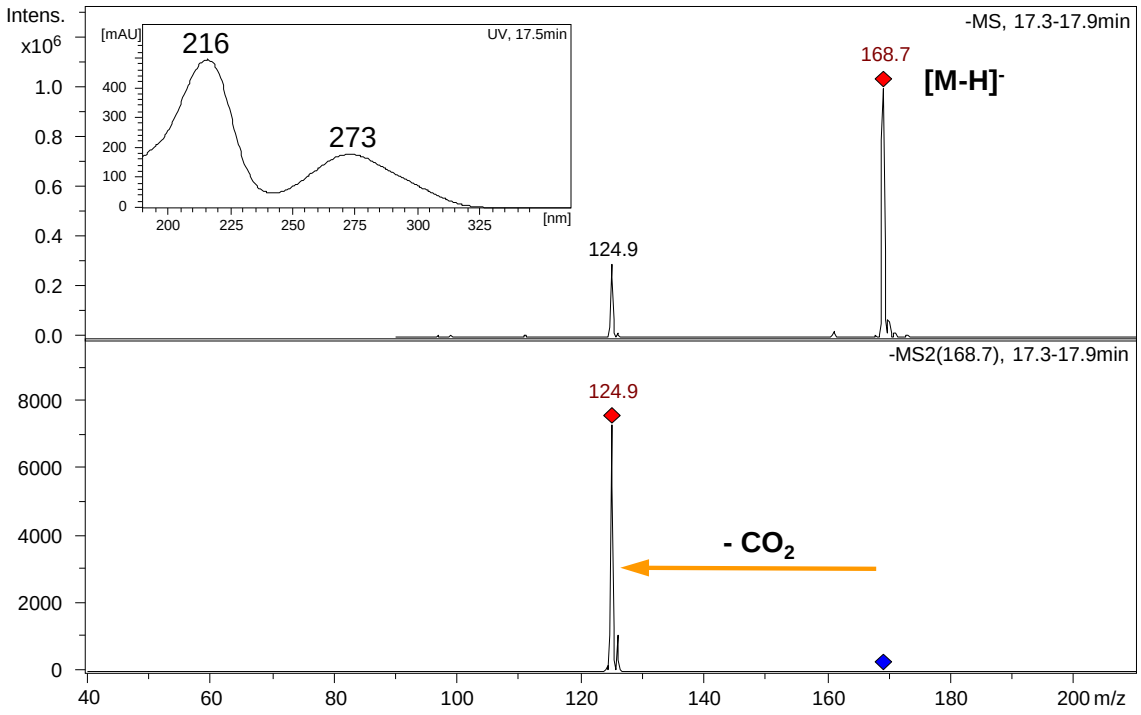


Figure 38: UV spectrum and MS-fragmentation pattern of gallic acid.

In addition, the eNOS activity of pure GA was determined. However, the three investigated concentrations, equal to these of the EG experiment (Figure 37), did not alter the eNOS activity at all compared to the control. Thus, the ethyl ester group seems to be the crucial structural requirement for the effect on eNOS enzyme, which is located in the EA.hy926 endothelial cells.

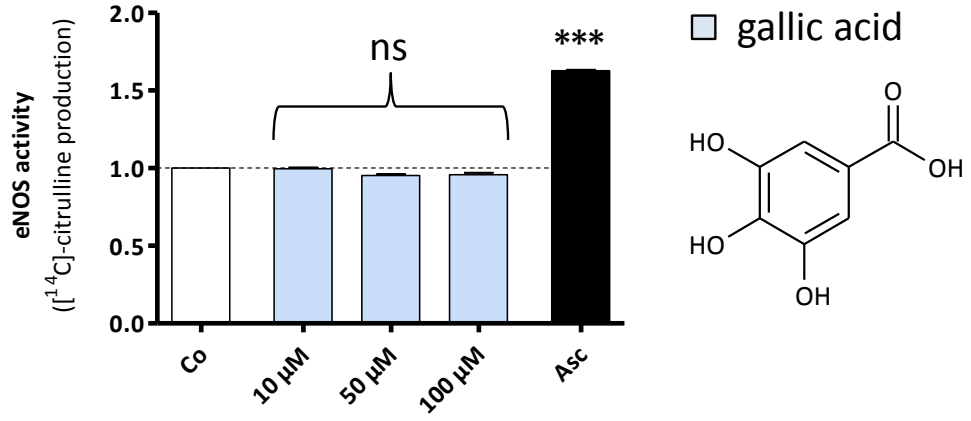


Figure 39: Gallic acid does not induce eNOS activation compared to the control level at 10 µM, 50 µM, and 100 µM concentration.

*EA.hy926 cells were treated with the indicated concentrations for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed. Control cells were incubated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) was used as positive control. [^{14}C]L-citrulline production was normalized to control (***, p < 0.001; ns, not significant) (duplicate, mean ± SEM, n = 3, ANOVA/Bonferroni).*

To support the actual findings, a third compound similar to gallic acid, which is also contained in red wine, was investigated. The 3,5-dimethylether of gallic acid is termed syringic acid (SA) . Due to the two methyl groups it has a very similar retention time as ethyl gallate (63.5 minutes vs. 65.0 minutes) using the HPLC-method A3. The only difference in the UV spectrum is a small bathochromic effect to 277 nm.

Both red wine components, SA and EG, are equal in their molecular weight of 198 g/Mol and occur as quasi-molecular ion at m/z 196.8 in the negative ESI-mode. But, the MS fragmentation pattern reveals differences as well as the MS^2 spectrum. The different binding sites of the substituents of the dimethylether and the ethyl ester represent a completely altered MS fingerprint, pictured in Figure 40 below and compared with Figure 35.

In particular, the MS^2 spectrum indicates fragments at m/z 181.7 and m/z 166.7 related to the loss of two methyl groups. The fragment at m/z 152.9 results from a loss of the carboxyl group, directly from the quasi molecular ion at m/z 196.8 in contrast to the CO_2 loss of EG.

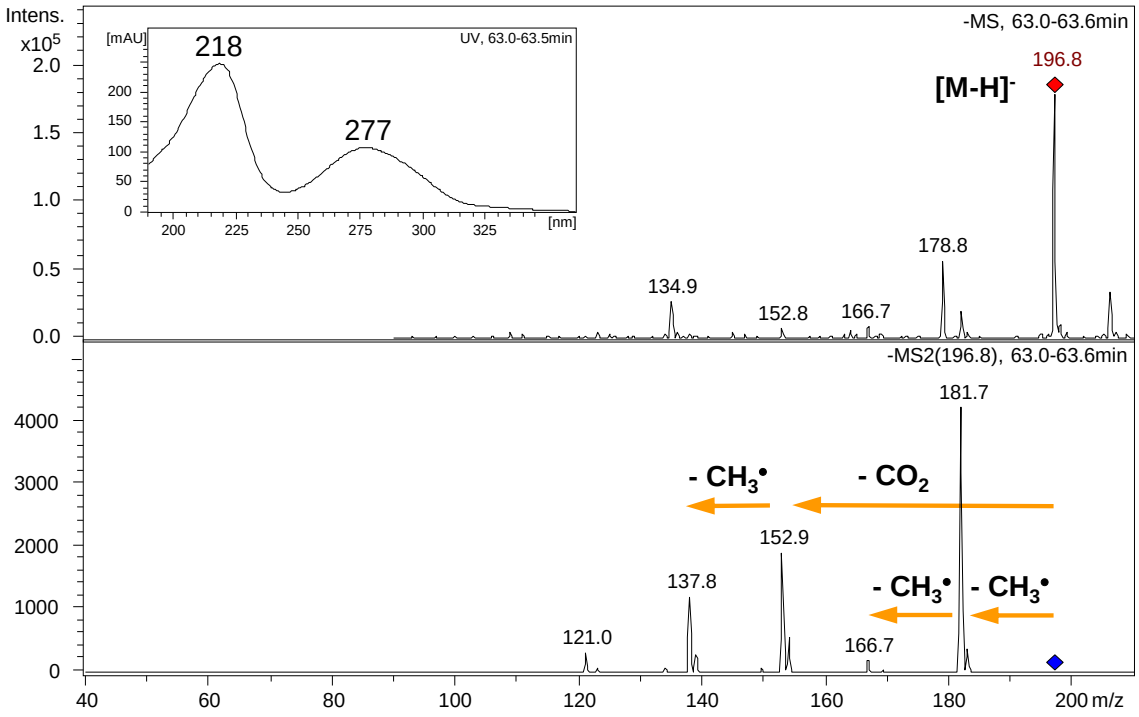


Figure 40: UV spectrum and MS-fragmentation pattern of syringic acid.

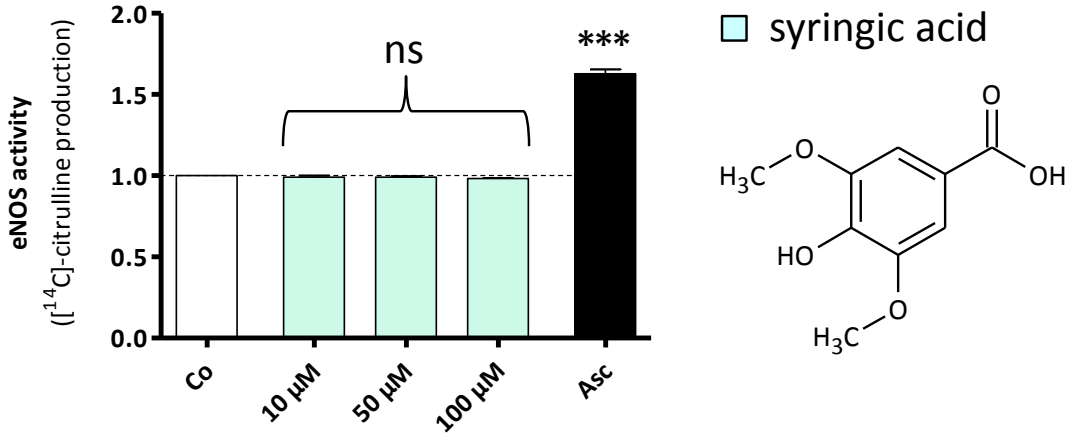


Figure 41: Syringic acid does not influence the [^{14}C]L-citrulline level from 10 µM until 100 µM concentration.

*EA.hy926 cells were incubated with the indicated concentrations for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed. Control cells were stimulated with DMSO (0.1 %), using ascorbic acid (Asc, 100 µM) as positive control. [^{14}C]L-citrulline production was normalized to control (***, $p < 0.001$; ns, not significant) (duplicate, mean ± SEM, n = 3, ANOVA/Bonferroni).*

SA was assayed as well at the three standard concentrations and did not modulate the eNOS activity anyway. Apparently, the methylations of the phenolic ring are not of importance for the enzyme activation. It can be assumed that the esterification initiates an activation of the eNOS enzyme.

3.1.6 *In vitro* generation of hydrogen peroxide has no influence on eNOS activation

Literature has indicated degradation of polyphenols under cell culture conditions and a formation of hydrogen peroxide, which might have influence on the result of the *in vitro* assay [135]. To prove that the measured eNOS activation by ethyl gallate, its analogs (see chapter 3.1.9), and RWPE was not depending on a modulation of the endothelial functionality caused by an altered H_2O_2 level in DMEM, the setup of the [14C]L-arginine/[14C]L-citrulline conversion assay was adapted. Additionally, confluent endothelial cells were pre-treated with catalase 10 minutes before conventional stimulation.

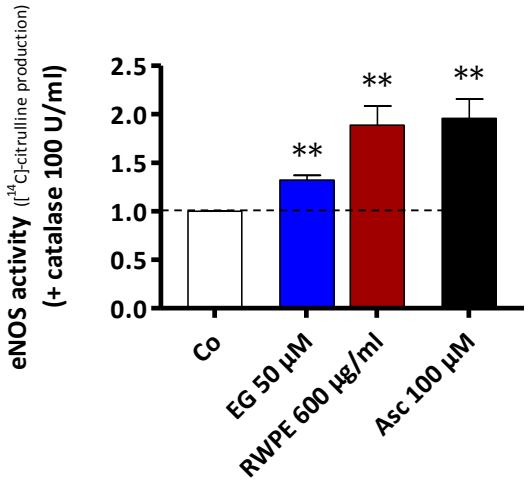


Figure 42: Addition of catalase demonstrates that the modulation of eNOS activity by red wine constituents develops independently from induced hydrogen peroxide formation.

*Endothelial cells were pre-treated with catalase (100 U/ml) 10 minutes before stimulation with the indicated polyphenols, 0.1 % DMSO (control), and 100 µM ascorbic acid (positive control). After 24 hours the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed. [^{14}C]L-citrulline production was normalized to control (**, $p < 0.01$) (triplicate, mean ± SD, n = 3, t-test).*

The result of the catalase assay demonstrated a comparable effect to the [14C]L-arginine/[14C]L-citrulline conversion assay after stimulation with ethyl gallate (see chapter 3.1.5) and RWPE (see chapter 3.1.1.3). Obviously, (possibly) generated hydrogen peroxide could not lead to a misinterpretation of the obtained results as no impact on the positive influence of red wine constituents on eNOS could be observed.

3.1.7 Influence of ethyl gallate on the eNOS mediated NO release

To support the received results of the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay about the increase of eNOS activity concerning the isolated red wine constituent EG, the DAF-FM assay, representing the measurement of released ("bioavailable") NO from the eNOS enzyme, was carried out (see chapter 2.6). In contrast to the eNOS activity measurement to quantify the intracellular NO level, the determination of the released NO aims at the extracellular level, and thus corresponds to bioavailable NO, needed in the physiological surroundings of endothelial cells (i.e. VSMC and lumen).

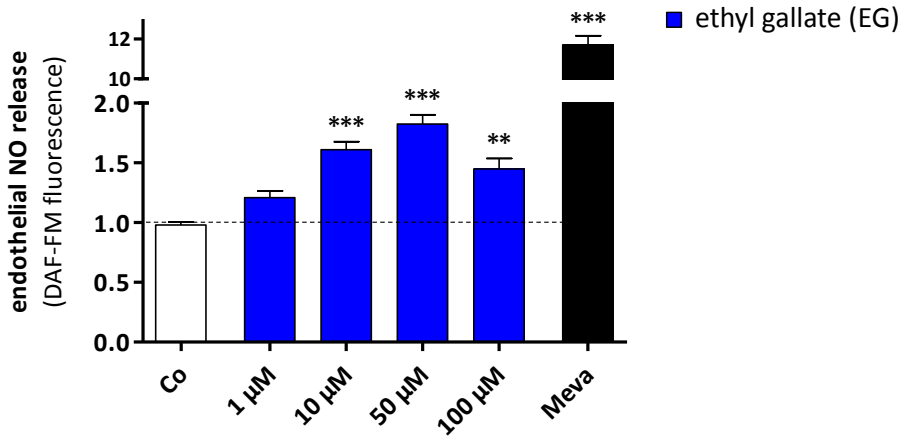


Figure 43: Ethyl gallate effects the endothelial NO release corresponding to its eNOS activity result (see Figure 37).

*Quantification of the endothelial NO release was employed by incubation with the NO-sensitive fluorescent probe DAF-FM. EA.hy926 cells were treated with for 24 hours. DAF-FM fluorescence was normalized to the cell count and to the activity of the control cells. Mevastatin (Meva, 3 µM) served as positive control. Control cells were stimulated with DMSO (0.1 %) (**, $p < 0.01$; ***, $p < 0.001$) (Five replicates, mean ± SEM, n = 4, ANOVA/Bonferroni).*

EG has already shown enhancement of the eNOS enzyme activity. Therefore, the influence of this eNOS-activating compound on the release of bioavailable endothelial NO was additionally measured. The result is in accordance with the outcome of eNOS activity (Figure 37), because a comparable activity pattern is given at 10 µM, 50 µM and 100 µM together with the highest influence at 50 µM and a decrease at 100 µM. Mevastatin was used as positive control (3µM) for the DAF-FM assay, showing a ten- to twelvefold NO release related to control.

3.1.8 Ethyl gallate leads to phosphorylation at Ser^{1177}

To gain further knowledge, the ability to influence the phosphorylation site at Ser^{1177} was investigated as well. Ser^{1177} phosphorylation is the most prominent post-translational regulatory mechanism of eNOS activity.

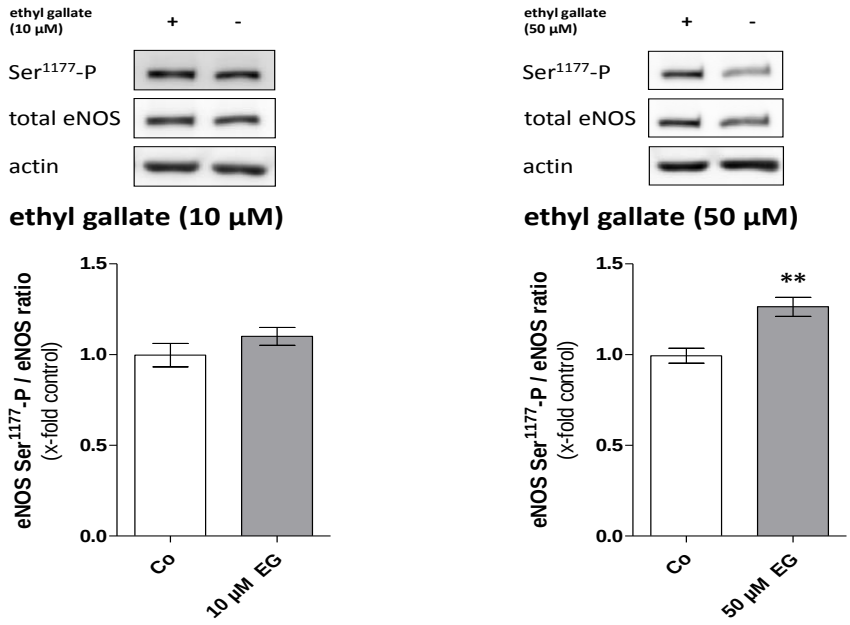


Figure 44: Ethyl gallate induces phosphorylation at eNOS-Ser^{1177}.

*EA.hy926 cells were treated ethyl gallate at the indicated concentrations for 48 hours. Cells were lysed, proteins were separated by SDS-PAGE and Western Blot analysis was performed for the quantification of eNOS Ser^{1177}, eNOS and actin. One representative blot is show. Band intensities were normalized to actin (**, p < 0.01; mean ± SEM, n = 3, t-test).*

Western blotting (chapter 2.7) allows a closer insight into the mechanism of eNOS activation, as the phosphorylation of Ser^{1177} is known to effect the enzyme activity enhancement. The experiment with 10 µM ethyl gallate did not show a significant increase of Ser^{1177} phosphorylation. But the 50 µM concentration induced an increase of Ser^{1177} phosphorylation after long-term treatment, which has to be associated to eNOS activation, and was even supporting the results of already performed experiments for this thesis.

3.1.9 Modulation of the endothelial nitric oxide synthase by different gallic acid esters in endothelial cells

The findings of the eNOS activity guided fractionation of Austrian red wine samples demonstrated gallic acid ethyl ester as major active compound. This raised question whether the nature of the esterified alcohol could have an impact on enzyme activation. Consequently, continued examinations concerning the influence of the alkylation on the enzyme activity were carried out to see a possible correlation between the length of the alkyl substituent at the carboxyl site and the level of eNOS activity (see chapter 3.1.5).

In this context, various alkyl gallates were provided to prove the structure-activity relationship of these esters to the endothelial nitric oxide synthase *in vitro,* employing the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay at concentrations ranging from 5 µM to 100 µM. Besides ethyl gallate, methyl, propyl, butyl, isopentyl, octyl, and lauryl ester were tested. Additionally, gallic acid was included to the measurement and used as control, as it could be already shown that this compound has no influence on the eNOS enzyme activity (Figure 38).

Figure 45 underlines the proof of our assumption. The gallic acid derivatives indicated to have an effect on the enzyme activity in contrast to gallic acid itself. Generally, an increase of activity could be observed in relation with the length of the alkyl substituent from the methyl up to the butyl ester, and it has to be emphasized that the effect on eNOS is coupled with the tested concentration as well. For instance, the

methyl ester shows a constant enhancement of eNOS activity from 5 µM to 100 µM, but butyl gallate reveals the maximum of eNOS activation already at 10 µM with a further decrease until 100 µM.

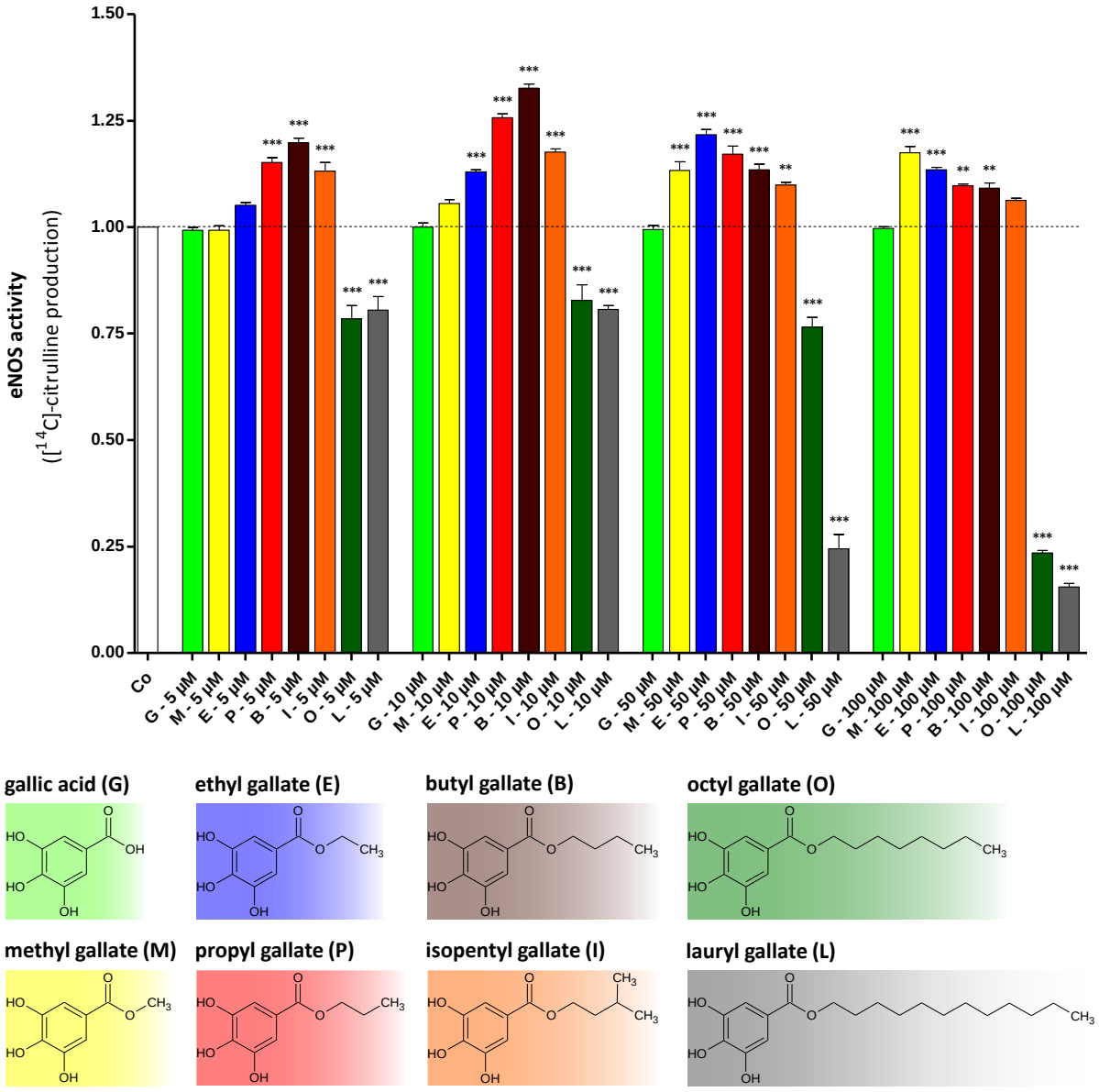


Figure 45: Gallic acid alkyl esters show correlation between the length of the alkyl substituent and the effect on the eNOS enzyme activity.

*EA.hy926 cells were stimulated with the indicated concentrations for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was carried out. Control cells were treated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) served as positive control. [^{14}C]L-citrulline production was normalized to control (**, $p < 0.01$; ***, $p < 0.001$) (duplicate, mean ± SEM, n = 3, ANOVA/Bonferroni).*

Moreover, it is remarkable that the activity response of this assay increases due to the alkyl residue from methyl over ethyl and propyl to the butyl residue (e.g. butyl gallate demonstrated the highest eNOS activation at the concentration of 10 µM compared to lower alkyl residues). Isopentyl gallate is only able to induce a smoother activity enhancement compared to butyl gallate, which seems to be affected by the aliphatic side-chain of the isopentyl residue. Octyl gallate and lauryl gallate caused a significant decrease of eNOS activity, which could be attributed to the microscopically observed disaggregation of the confluent, viable *in vitro* monolayer of endothelial cells, at least at 50 µM (lauryl gallate) and at 100 µM (octyl and lauryl gallate).

3.1.10 Red wine vinification impacts the level of eNOS activity

To get information about the impact of red wine fermentation regarding the modulation of the enzyme activity, a vinification experiment was performed. The mash of Merlot grapes was provided by a winery (B. Wiederstein, Göttlesbrunn, Lower Austria) in September 2007, recently after preparation. It was fermented immediately for 15 days in the laboratory of the Department of Pharmacognosy in Vienna following the instructions of the winemaker. The process was recorded in a fermentation protocol, and the notes are shown in Table 19 including the date of sample drawing (samples were defined M1 – M5, representing the status of mash fermentation), the density of the liquid (representing the increase of ethanol), a description of taste, and data concerning the volume and the weight of the mash samples. The drawn sample volumes were refilled using ddH_2O to avoid enrichment of the components. The density measurement was performed with a hydrometer (alcoholometer). The calculated final concentration of ethanol was about 13 % (v/v). The description of taste is based on a personal organoleptic perception.

	MASH 1		MASH 2			MASH 3
Date	2007/09/14 1st day after mashing	2007/09/17	2007/09/18 5th day after mashing	2007/09/19	2007/09/20	2007/09/21 8th day after mashing
Density (ρ) at 20 °C	1.098 (1.09 kg/l)	1.035	1.007 (1.01kg/l)	1.001	1.000	1.000 (1.00 kg/l)
Character of taste	Supernatant can be compared to grape juice → very sweet, no alcoholic taste		Vinification process has started, when stirring the mash it gets the typical smell of fermentation, taste at the beginning slightly alcoholic, final note very sweet			Alcoholic taste, final taste less sweet

	MASH 4			MASH 5
Date	2007/09/24 11th day after mashing	2007/09/25	2007/09/26	2007/09/27 14th day after mashing
Density (ρ) at 20 °C	0.999 (0.99 kg/l)	0.999	0.998	0.998 (0.99 kg/l)
Taste character	Alcoholic taste, no sweet final taste any more			Alcoholic taste, no sweet final taste any more, slightly sour

Table 19: Fermentation protocol indicates the recorded data during the vinification process representing the mash samples M1 – M5.

Five samples were taken during the vinification period. To study, whether the increase of ethanol, which comes along with a better extraction of compounds – especially of more lipophilic ones – is coupled to the ability to improve the eNOS activity of EA.hy926 endothelial cells [69, 136], these mash samples (defined as M1 – 5) were assayed at three concentrations (Figure 46). M1 can be described as pure grape juice (Table 19), when compared to the other Mash samples. Generally, there is an evident trend that starting with M1 up to M5 the activation of eNOS develops, related to the fermentation process, in an increasing manner. Additionally, dose-dependency can be observed. The juice of these Merlot grapes shows an effect at 50 µl/ml and 100 µl/ml, pointing out that even the juice of red grapes contains activating compounds.

However, the main focus of this diagram was to emphasize the increasing effect monitored during vinification, which could be explained by biochemical synthesis of relevant components due to fermentation (e.g. ethyl gallate).

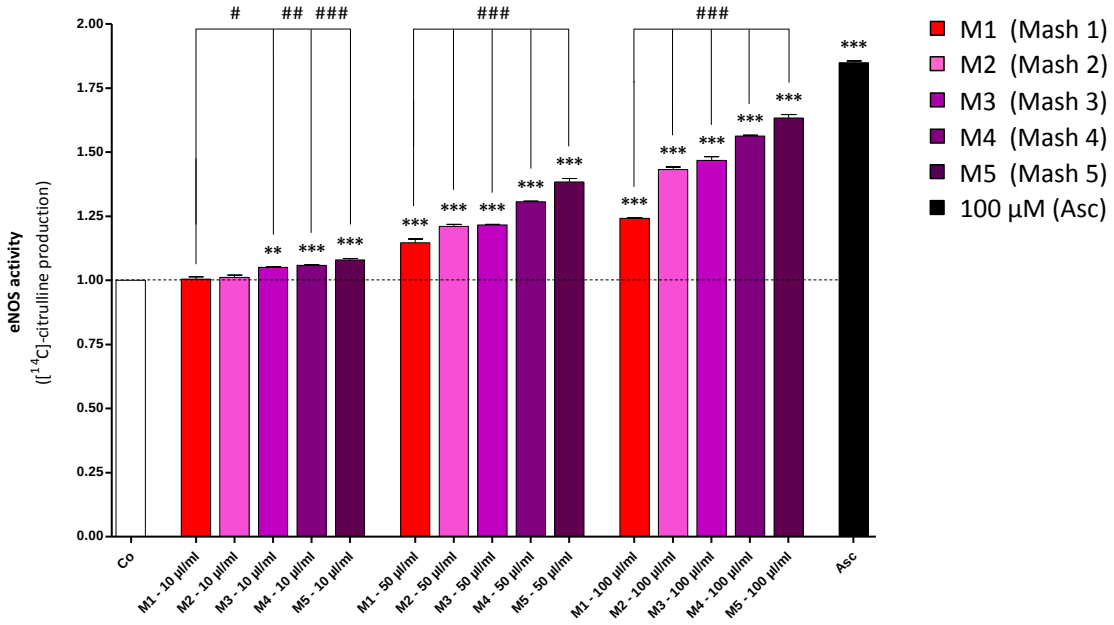


Figure 46: The vinification process indicates influence on eNOS activity enhancement, and furthermore dose-dependency.

*EA.hy926 cells were incubated with the shown concentrations for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed. Control cells were stimulated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) served as positive control. [^{14}C]L-citrulline production was normalized to control (**, p < 0.01; ***, p < 0.001; #, p < 0.05; ##, p < 0.01; ###, p < 0.001) (triplicate, mean ± SEM, n = 3, ANOVA/Bonferroni).*

To elucidate the fact that M5 showed a higher activity than M1, a comparison of the HPLC-analyses was employed (method A2 in chapter 2.3.1). According to the former results of the eNOS activity guided isolation leading to ethyl gallate as main active compound (3.1.4.2), the HPLC-system was established to selectively quantify and compare its amounts in the mash samples.

By the way, the remaining mature part of sample M5 was filtered, bottled and stored at 4 °C in a refrigeration room for about three years until December 2010. This “wine” was termed M6 for a further analysis, where the mash samples M1 – M5 together with M6 were compared for their ethyl gallate content in order to screen the development of this compound during vinification.

The comparison of HPLC-chromatograms in Figure 47 demonstrated that the obtained amounts of ethyl gallate were consistent with the results of the eNOS activity

measurement of M1 – 5 (Figure 46). M1 did not contain ethyl gallate at all, but starting with M2 a permanent increase of this eNOS-activating compound could be observed.

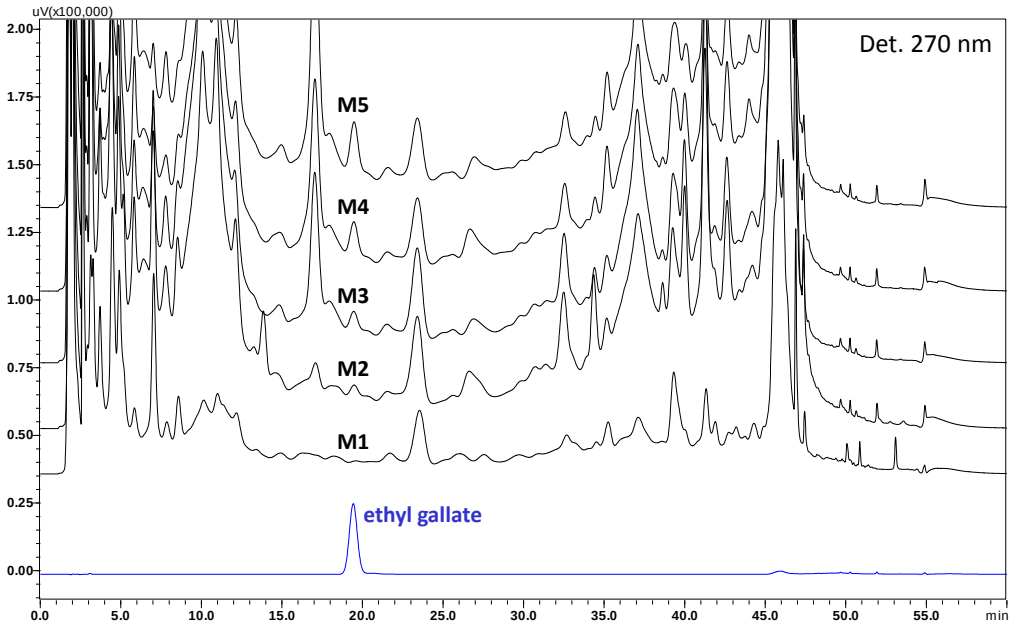


Figure 47: Quantitative determination of ethyl gallate in Mash 1 – 5 using HPLC, detected at 270 nm (method A2; chapter 2.3.1).

The content of this gallic acid ethyl ester was also quantified in sample M6 expecting an increased amount of the compound with regard to the storage time of three years. To obtain the exact concentrations of ethyl gallate of the occurring mash samples together with M6, a calibration curve of ethyl gallate was performed in a concentration range from 1 µM to 100 µM (i.e. 0.2 µg/ml to 20 µg/ml), presented in Figure 51.

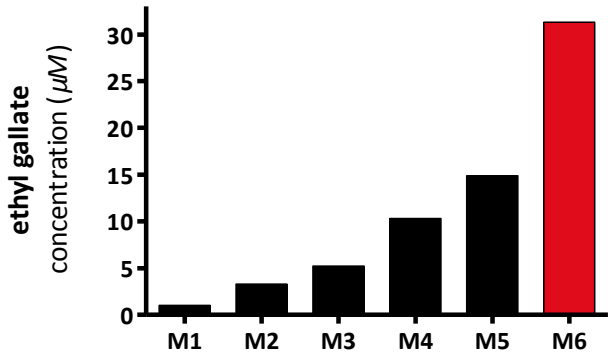


Figure 48: Increase of the ethyl gallate concentration in red wine during vinification, quantified with the analytical HPLC-method A2 at 270 nm (chapter 2.3.1).

The analysis above (Figure 48) is underlining the result of Figure 47 . The quantity of ethyl gallate is depicted in a further diagram to express the increase of ethyl gallate, which is depending on the fermentation. The gallic acid ethyl ester formation processed analogously to the fermentation with a scale ranging from a 1 µM concentration in M1 to about 15 µM in M5. Moreover, the quantity of the compound augmented during the storage for three years (M6) to the twice as high concentration of about 31 µM.

3.1.11 Sample of typical Austrian white wine does not improve the eNOS activity

To check whether Austrian white wine has influence on the eNOS as well, a typical white wine sample from Austria – a "Grüner Veltliner 2006" (GV; see section 2.1.4) – was tested using the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay. Identical concentrations related to red wine analyses (50 µl/ml and 100 µl/ml, Figure 24; 600 µg/ml, Figure 14 and Figure 16) were taken for stimulation with white wine as well.

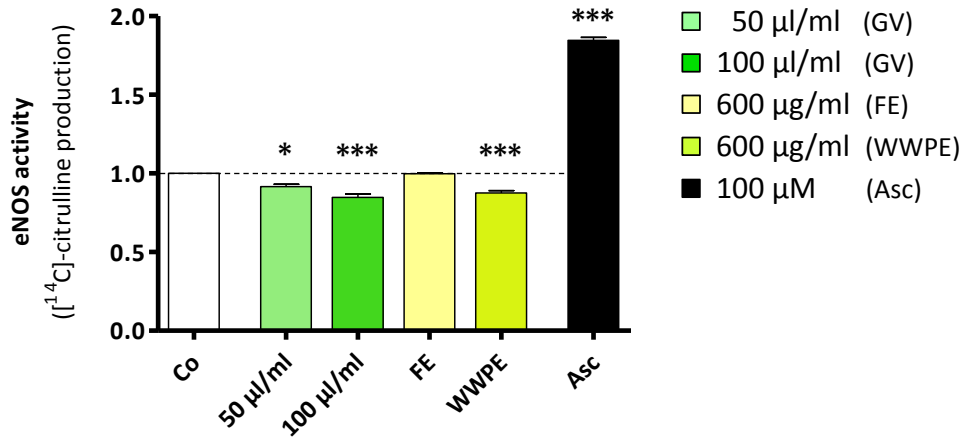


Figure 49: One sample of "Grüner Veltliner" (GV), a typical Austrian white wine, shows a decreasing effect on eNOS activity.

EA.hy926 cells were stimulated with the indicated concentrations for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was employed. Control cells were treated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) was the positive control. [^{14}C]L-citrulline production was normalized to control (, $p < 0.05$; ***, $p < 0.001$) (triplicate, mean ± SEM, $n = 3$, ANOVA/Bonferroni).*

The assayed samples demonstrated a slight, significant decrease of eNOS activity in contrast to the red wine results. The only sample, which had neither an inhibiting nor an activating effect on eNOS, was the FE.

This white wine sample did not to contain relevant amounts of the isolated eNOS-activating red wine constituent ethyl gallate as shown in chapter 3.1.12.

3.1.12 Quantitative comparison of ethyl gallate in various wine samples

To find out the ethyl gallate content of different Austrian red wine samples, a HPLC-comparison was performed. For this analysis the analytical HPLC-method A2 was applied (section 2.3.1). The chromatogram in Figure 50 represents the analysis of the pure compound with a retention time of 19.5 minutes at a detection wavelength of 270 nm (chapter 3.1.5). In the background of the HPLC-chromatogram the gradient of the used method is additionally displayed.

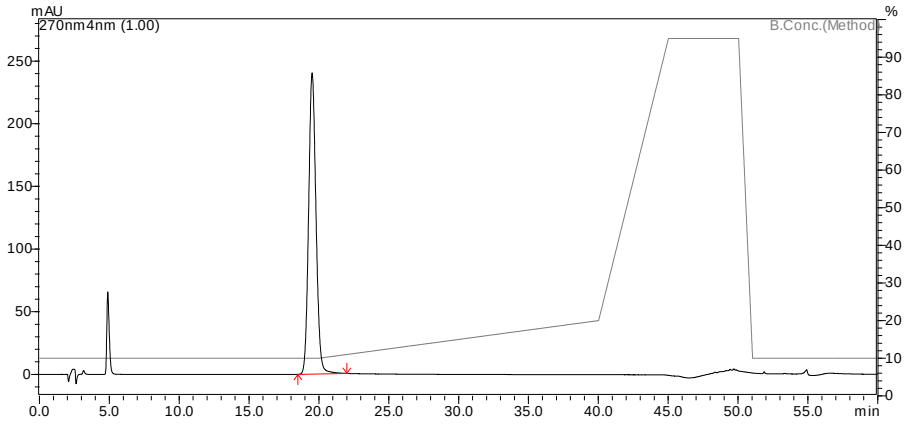


Figure 50: Typical HPLC-chromatogram of pure ethyl gallate, analyzed with method A2 (chapter 2.3.1), detected at 270 nm for the quantification in wine samples.

Furthermore, the calibration curve of EG is depicted below, quantified at a range from 0.2 µg/ml to 20 µg/ml, which is equal to concentrations from 1 µM to 100 µM, used for the bioassays, regarding eNOS activity and NO release (Figure 37 and Figure 43). The correlation coefficient (r^2) reached 0.9996 indicating a sufficient linearity for the quantification. The coefficient of variation (CV) was between 0.28 % and 7.04 %. Each standard (red marks on the calibration curve) represents the mean value of five measurements.

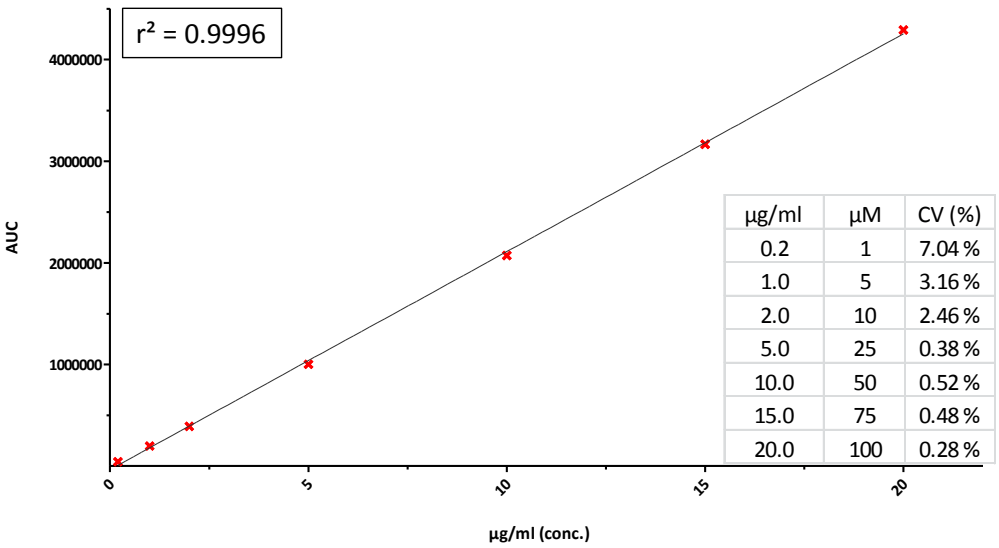


Figure 51: Calibration curve of ethyl gallate, quantified at 270 nm with method A2 (chapter 2.3.1).

Eight wine samples, which were all investigated for the influence on eNOS activity, were quantified for their EG content (chapter 3.1.1.3., 3.1.4.1 and 3.1.11).

While all of the red wine samples were containing ethyl gallate, the responsible peak was below the LOD in the white wine sample (Figure 52).

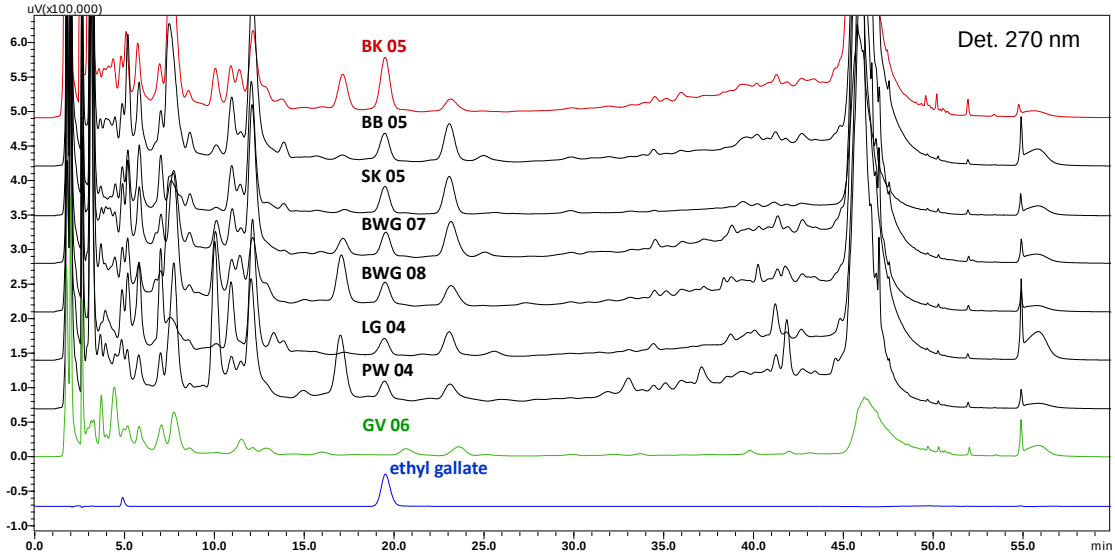


Figure 52: Seven red wine samples and one white wine sample are quantified for their ethyl gallate content at 270 nm (method A2; chapter 2.3.1).

In accordance to the latter results, the EG quantities of the seven red wine samples were correlated to their eNOS activity to verify the relation (see Figure 53 below). The diagram shows three clusters, two of them representing three ethyl gallate quantities in red wine; one varies from concentrations of 20 µM to 25 µM concerning the samples PW 04, LG 04 and BWG 08. The other one illustrates the amounts of BWG 07, SK 05 and BB 05 ranging from 30 µM to 35 µM. The red wine sample BK 05 reveals and outstanding concentration of ethyl gallate, which is approximately 68 µM (twice as high EG concentration as the other samples). The BK 05 is the red wine sample, which showed the highest eNOS activation in the Second Test Series (3.1.2.2) and was therefore chosen to get fractionated in the Third Test Series (3.1.3.2). Moreover, this red wine sample was selected for the activity guided fractionation regarding the inhibition of *Chlamydia pneumoniae*, examined in chapter 3.2.

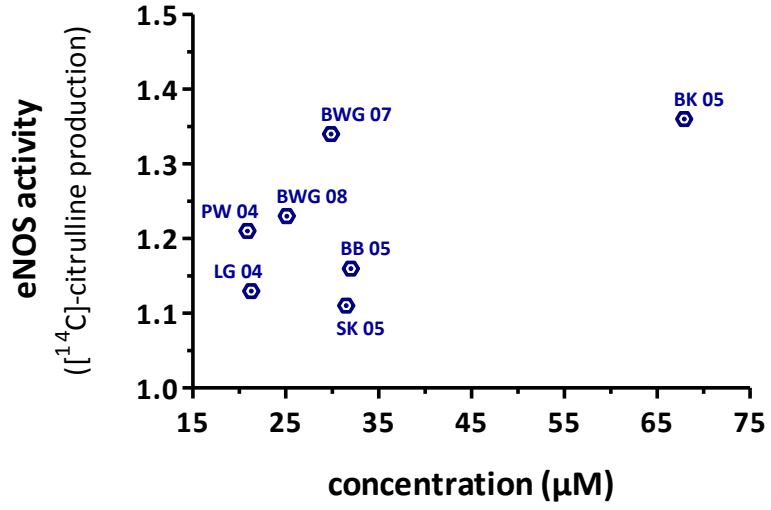


Figure 53: Scatterplot representing the ethyl gallate concentration of seven red wine samples, related to their eNOS activation level.

Due to deviation in the increase of eNOS activity of the seven analyzed samples, which does correlate partially with the measured concentrations of eNOS activating ethyl gallate, this compound could not be exclusively associated to the influence on this enzyme. But, ethyl gallate could be regarded as marker for the eNOS activation of red wine and indicates antiatherosclerotic potential.

A complexity of polyphenols in red wine seems to be responsible for the complete eNOS activation of red wine, which could now be elucidated in part by the findings of this thesis.

3.1.13 eNOS activation is not affected by red wine storage in barrique

The process of red wine maturation in oak barrels always gets along with the extraction of wood substances like ellagitannins, which have at least an impact on the roundness and amplitude of the organoleptic perception of red wine [137]. Previous investigations concerning the human eNOS promotor showed an activity decrease [69]. To figure out, whether ellagitannins alter the eNOS enzyme activation, a completely fermented Merlot 2007 (see chapter 2.1.5) was stored for six month in two different wine casks, either in stainless steel or in barrique. The first samples were taken in December 2007, 5 days after transferring the wine into both casks. The second samples were drawn just before bottling the Merlot 2007, in June 2008. To test the effect of “barrique compounds”, the setup for the assay was created comparing both the stainless steel and the barrique storage at two specific dates. The Merlot samples were used for stimulation of the EA.hy926 endothelial cells at concentrations of 10 µl/ml, 50 µl/ml, and 100 µl/ml.

The result of the samples of December 2007 showed a similar (dose-dependent) effect, comparable to former testings (Figure 24 and Figure 46) without any deviation between stainless steel and barrique storage.

An organoleptic testing of the barrique sample of December 2007 exhibited a noticeable smoother taste after five days, which set it already apart from its counterparts taste. However, keeping the red wine just for a few days in the different wine casks was not enough time to reveal a deviation of eNOS enzyme activation at any tested concentration (Figure 54, left side).

Finally, after six months of maturation, there was still no significance in modulation of the enzyme activity detectable at the applied concentrations neither of barrique nor stainless steel storage within six month (Figure 54, right side), concluding that the barrique compounds do not influence eNOS activity.

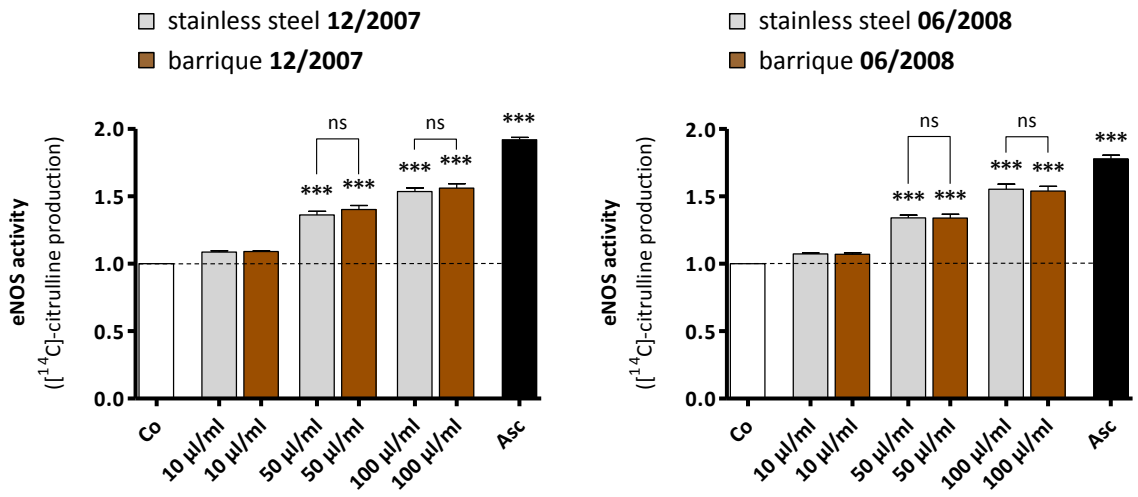


Figure 54: Storage of a Merlot 2007 in barrique does not influence the potential of the red wine on eNOS activity compared to stainless steel storage at any tested concentration.

*EA.hy926 cells were incubated with the indicated concentrations for 24 hours and a [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed. Control cells were stimulated with DMSO (0.1 %), ascorbic acid (Asc, 100 µM) served as positive control. [^{14}C]L-citrulline production was normalized to control (***, $p < 0.001$; ns, not significant) (quadruplicate, mean ± SEM, n = 3, ANOVA/Bonferroni).*

3.2 Red wine as natural agent against *Chlamydia pneumoniae*

Chlamydia pneumoniae is a bacterium primarily causing infections of the respiratory tract, but is discussed intensively for more than twenty years for its impact on the development of atherosclerosis as well [97]. Due to the exceptional chlamydial life-cycle it is able to form persistent inclusions, which are not treatable sufficiently with a common therapy using antibiotics. *C. pneumoniae* gains access to the vasculature through the endothelial cell layer into the intima and is infecting both. Therefore, the endothelial cells express pro-inflammatory cytokines, leukocytes migrate through the endothelial cell layer to induce the formation of lesions in the intima, and consecutively the development of atherosclerotic plaque is provoked [104] (see Figure 5).

Meanwhile, some polyphenols, which are generally associated with a positive effect targeting inflammation, are known to inhibit *C. pneumoniae* infection [120]. Thus, even red wine as a polyphenol-rich source is likely to influence this kind of infection, and hence a bioassay guided fractionation of two Austrian red wine samples was performed to isolate antichlamydial-acting components.

This part of the thesis will present data about red wine and isolated compounds as agents acting against *C. pneumoniae* infection.

Section 3.2.1 deals with the fractionation of two selected Austrian red wine samples including the assaying of obtained fractions, followed by further antichlamydial testings of ten subfractions (3.2.2) and a *C. pneumoniae* growth inhibition by identified pure compounds in section 3.2.3. The inhibition of chlamydial inclusions of ethyl gallate, which was isolated from red wine, is subject of chapter 3.2.4.

3.2.1 Fractionation of red wine compounds to inhibit *Chlamydia pneumoniae*

Based on the red wine data, which were obtained performing the First and the Second Test Series to measure eNOS activity (see chapters 3.1.1 and 3.1.2), the two most promising red wine samples were chosen for bioactivity guided fractionation. To find

out, whether red wine from Austria has an inhibiting effect on *Chlamydia pneumoniae* infection, the BK and the ZC (section 3.1 and Table 18) were examined.

The two wines were fractionated according to the schemes in Figure 12 and Figure 17 to yield four fractions: The First Eluate (FE), the Red Wine Phenol Extract (RWPE), the Polar Fraction (PF), and the Apolar Fraction (AF).

They were tested for the inhibition of the *C. pneumoniae* growth at a concentration of 40 µg/ml (illustrated in Figure 55), measured with the TR-FIA method (chapter 2.8.2). Generally, the red wine fractions of BK and ZC revealed a comparable activity pattern, but the BK samples reached higher inhibition (57 % – 84 %) matched to the ones of ZC (16 % – 62 %). Finally, the most efficient fraction was the AF of BK, and it was chosen for continued fractionation, combined with antichlamydial assays representing both growth inhibition and eradication potential of *C. pneumoniae* inclusions.

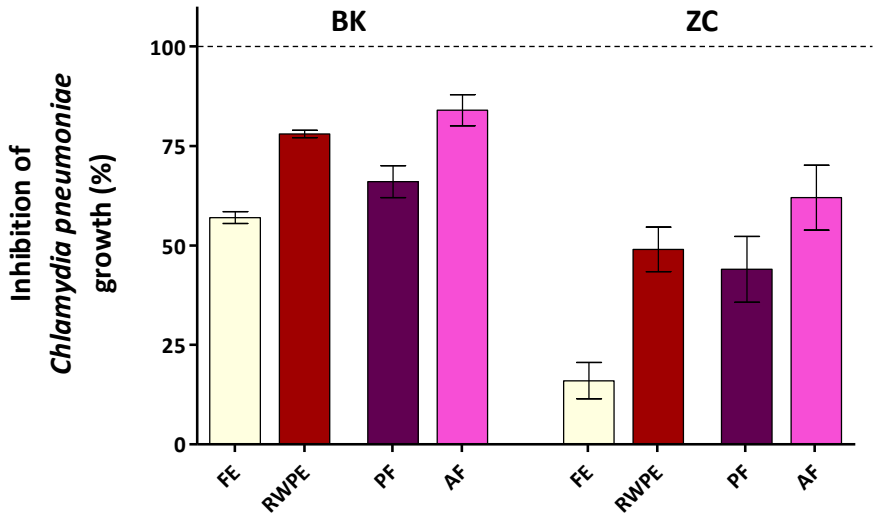


Figure 55: Inhibition of chlamydial growth and host cell viability of fractions of BK and ZC at 40 µg/ml.

HL cells were incubated with the indicated concentration (40 µg/ml) for 72 hours. The measurement was performed with the TR-FIA method. The inhibition of chlamydial growth is presented as percentage (n = 6, mean ± SEM).

There was no indication of toxicity to host cells, as all fractions and all compounds used in this study were well tolerated (Figure 55). The host cell viability was determined with the ATPase-based CellTiter-Glo® Luminescent Cell Viability Assay (chapter 2.8.4) and was between 94 % and 97 % within these experiments (data not shown). The wine

fractions were also tested for autofluorescence and binding of the TR-FIA label, and no disturbing effects on the measurement method were observed (data not shown).The fraction AF of BK was further separated using the semipreparative HPLC-method P2 (chapter 2.2.5), and HPLC/MS-method A3 (chapter 2.3.2) was employed to identify main components of the AF (depicted in Figure 56) in order to test pure reference substances with the corresponding fractions in parallel for their inhibition of *C. pneumoniae* (Figure *57* and Figure 58).

Ten subfractions (F1 – F10) were collected from the AF of BK. Most of them contained at least one of ten identified compounds. Exception was F4 with two identified compounds. F10 consisted of longer retained components (such as flavonoids and stilbenes), which were not further characterized.

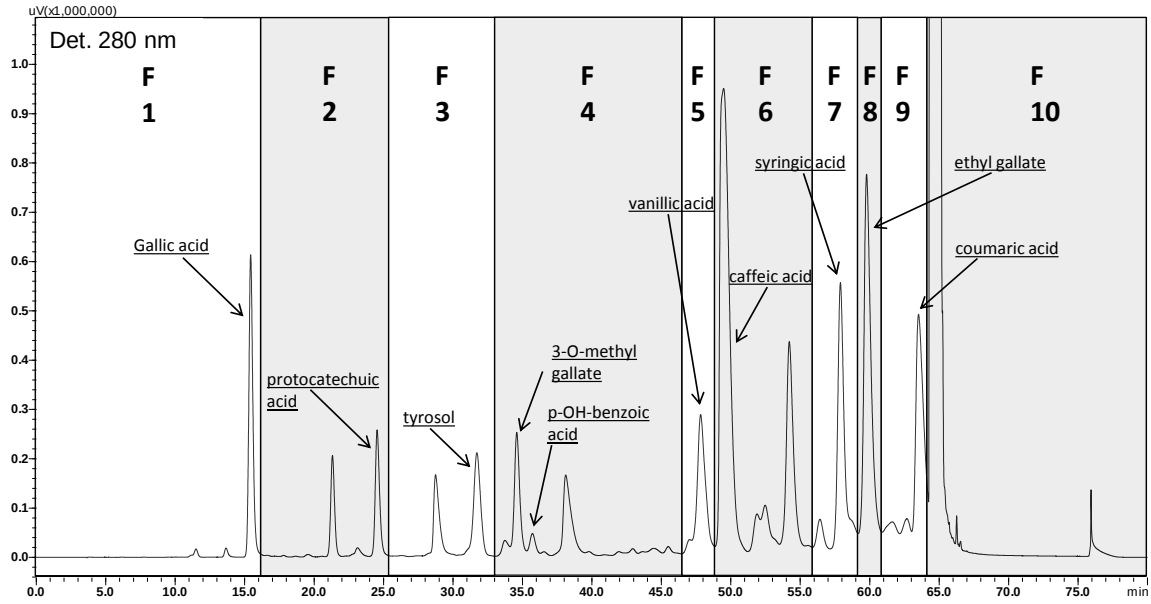


Figure 56: HPLC-chromatogram representing the ten subfractions F1 – F10 of AF (BK) indicating identified compounds, detected at 280 nm (method P2; 2.2.5).

3.2.2 Comparison of inhibiting effect of subfractions F1 – F10 and related pure compounds on *Chlamydia pneumoniae* inclusions

The ability to inhibit inclusions of *C. pneumoniae* was compared between subfractions (F1 – F10) and reference substances of identified main constituents (see Figure 57)

using an immunofluorescence method (chapter 2.8.3). Host cell viability lay between 97 % and 107 % concerning these experiments (data not shown).

Ethyl gallate showed an almost complete inhibition of the bacterium similar to the subfraction F8 containing ethyl gallate. The quantity of ethyl gallate in the 40 µg/ml concentration for the assaying of the subfraction was estimated to correspond at the utmost to a 200 µM concentration of the pure substance.

The subfractions F1, F5, and F7 contained primarily the single compounds gallic acid, vanillic acid, and syringic acid, respectively. The antichlamydial activity of these subfractions was higher than the effect of the pure compounds (50 µM), as the subfractions inhibited *C. pneumoniae* growth by 66 %, 79 %, and 28 %, and the compounds by 44 %, 28 %, and 0 %, respectively.

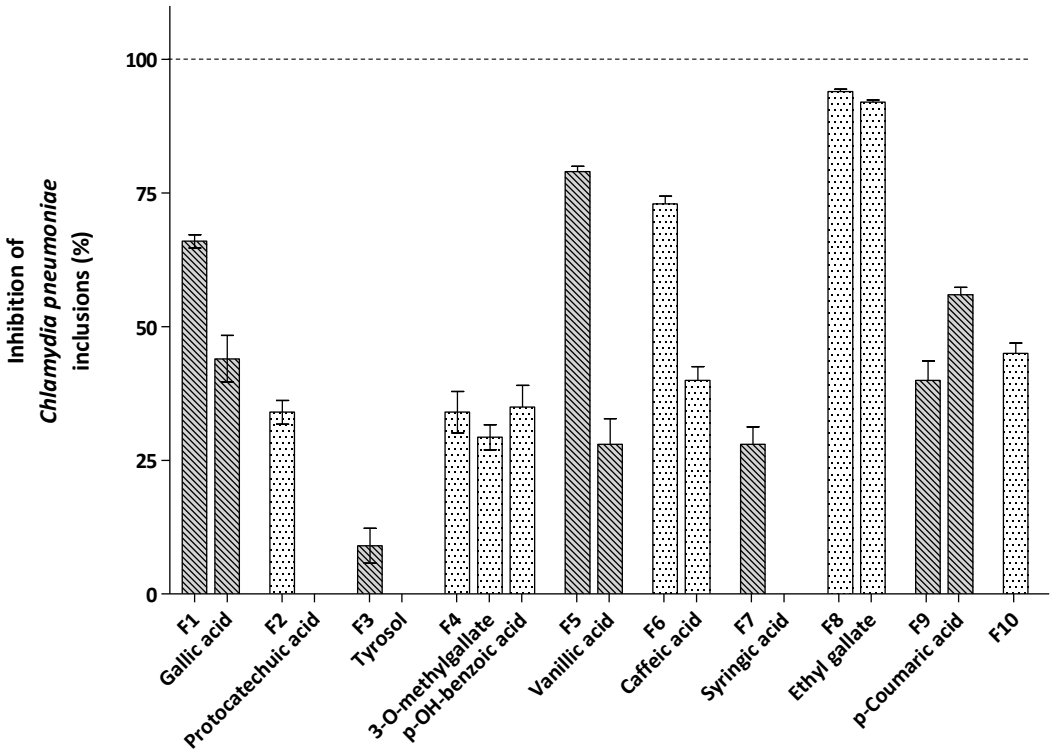


Figure 57: Comparison of the inhibition potential of Chlamydia pneumoniae inclusions by the subfractions of the AF (40 µg/ml) and related, identified main compounds (50 µM).

HL cells were stimulated at refered concentrations for 72 hours. The assay was employed with the immunofluorescence microscopy method for inhibition studies and host cell viability. The inhibition of chlamydial inclusions is shown as percentage (n = 6, mean ± SEM).

Caffeic acid (F6) corresponded as well in some degree with the associated effect (F6). F2 and F3 showed a slight inhibition, their related compounds protocatechuic acid and tyrosol were inactive. Subfraction F4 revealed only 34 % inhibition whereas both identified constituents, 3-O-methyl gallate and p-OH-benzoic acid, were as potent at 50 µM as 40 µg/ml of fraction F4. P-coumaric acid was not the main compound in subfraction F9, but inhibited chlamydial growth by 52 % (subfraction F9 was not comparably effective as p-coumaric acid). F10 represented the remaining bulk of red wine constituents, which were not further characterized regarding this examination.

3.2.3 Inhibition of *Chlamydia pneumoniae* growth by pure compounds

The antichlamydial effect of main compounds identified in the subfractions and further prominent red wine components – *trans*- and *cis*-resveratrol, (-)-epicatechin and (+)-catechin – were assayed with the TR-FIA method (chapter 2.8.2). The host cell viability concerning these experiments was between 99 % and 108 % (data not shown). Ethyl gallate (50 µM) yielded the most outstanding result, showing an almost quantitative inhibition of *Chlamydia pneumoniae* growth (97 %; see Figure 58), which was comparable to the effect of the control antibiotic rifampicin at 12 nM (93 %), and even more remarkable than three of the additionally tested components (*trans*-resveratrol, *cis*-resveratrol, and (-)-epicatechin) demonstrating a growth inhibitory effect of about 75 %. Besides ethyl gallate, which was identified in subfraction F8, some further identified red wine components showed a growth inhibition of approximately 50 % at a concentration of 50 µM. These were protocatechuic acid (F2), 3-O-methylgallate (F4), p-OH-benzoic acid (F4), and syringic acid (F7).

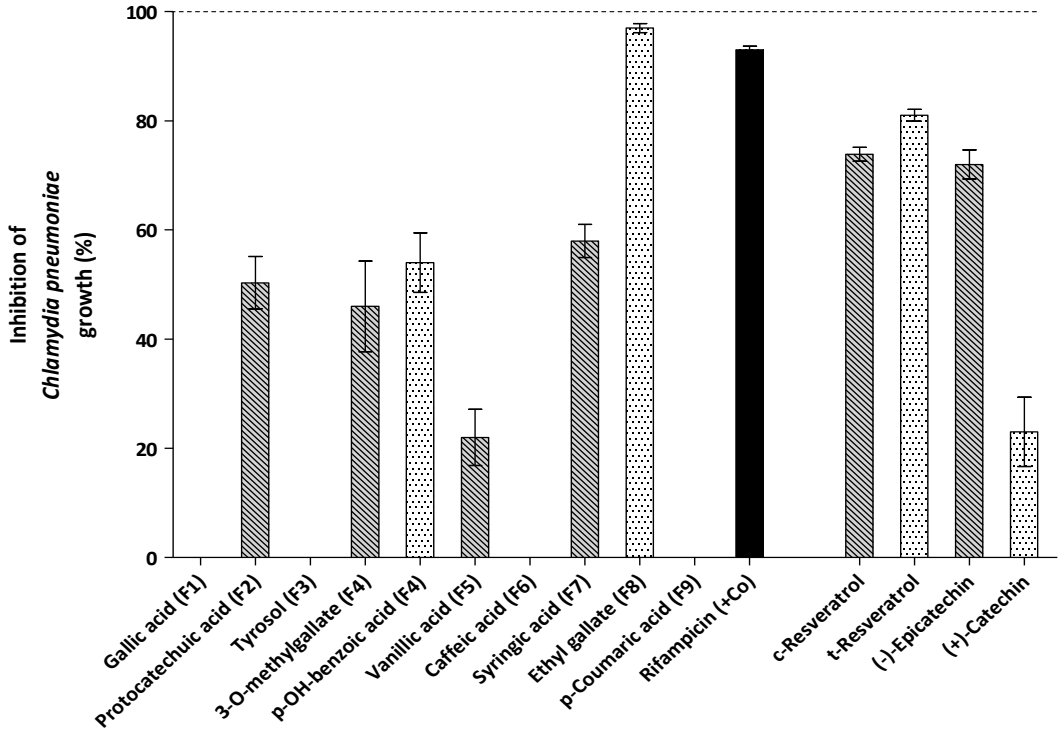


Figure 58: Inhibition of chlamydial growth of major phenolic compounds, identified in AF of BK 05 and tested at 50 µM.

HL cells were treated with the shown concentration for 72 hours. The measurement was carried out with the TR-FIA method for both the inhibition and the host cell viability. The inhibition of chlamydial inclusions is shown as percentage (n = 6, mean ± SEM).

3.2.4 Inhibition of *Chlamydia pneumoniae* inclusions by ethyl gallate

As a result of the activity guided fractionation, the dose-dependency of subfraction F8 was determined in parallel with the effect of pure ethyl gallate for the potential to decrease inclusions. Moreover, the gallic acid ethyl ester was the most potent inhibitor of *C. pneumoniae* growth (Figure 58).

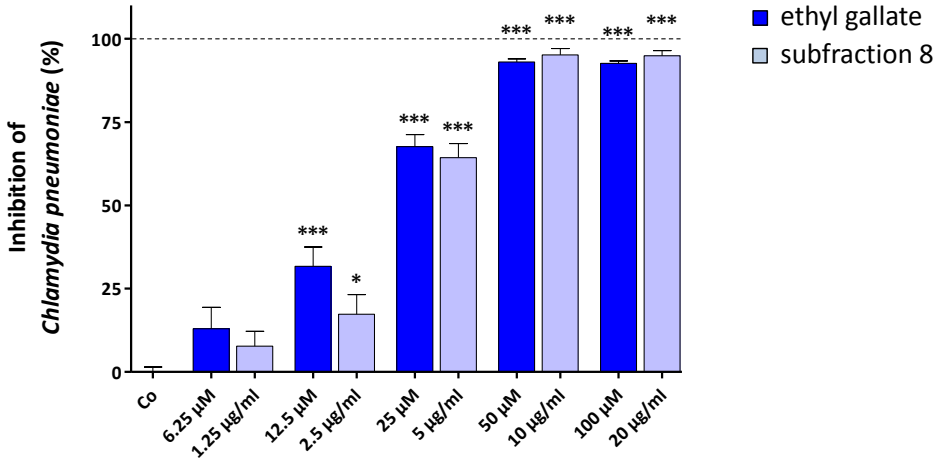


Figure 59: Inhibition of Chlamydia pneumoniae inclusions by ethyl gallate compared to subfraction F8 of the AF of BK.

HL cells were incubated with the shown concentrations for 72 hours. The assay was performed with the immunofluorescence microscopy method for the inhibition studies and the host cell viability (102 %; data not shown). The inhibition of Chlamydia pneumoniae (n = 10, mean ± SEM) is presented as percentage compared to non-treated control (, p < 0.05; ***, p < 0.001) (ANOVA/Bonferroni).*

As illustrated in Figure 59 above, ethyl gallate was also inhibiting the formation of inclusions completely at 50 µM (MIC) and 100 µM. The IC_{50}-value was approximately 20 µM. The Inhibition of F8 was similar to the effect of ethyl gallate as pure compound with the estimation of 1 µg/ml of this subfraction F8 to correspond to 5 µM of ethyl gallate.

Several polyphenolic components of red wine have demonstrated the ability to inhibit the growth of *C. pneumoniae* together with eliminating inclusions *in vitro*. The most striking isolated constituent was ethyl gallate, which was already confirmed in the first part of the thesis to be the main eNOS activator. Ethyl gallate showed a quantitative inhibition of *C. pneumoniae* growth as well as eradication of inclusions at a concentration of 50 µM.

As shown in chapter 3.1.12, pure red wine is able to contain relevant amounts of ethyl gallate (e.g. sample BK 05).

4 DISCUSSION

Atherosclerosis is a complex disease and represents the precursor for CVDs. Endothelial dysfunction and *C. pneumoniae* infection are not the only causes provoking develop-ment of atherosclerosis, but both are well described as contributing risk factors (see chapter 1.2. and chapter 1.4).

Results of this thesis illustrate the isolation and identification of ethyl gallate from red wine using activity guided fractionation, and demonstrate its multipotent character *in vitro*.

4.1 Wine and eNOS

The proof of enhanced NO production is the key point of eNOS cascade experiments, and the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay represents the key experiment. This assay to measure the eNOS enzyme activity *in vitro* provides the essential results of the eNOS cascade experiments (see chapter 1.5.1). It proved to be an adequate tool to perform an activity guided fractionation in conjunction with state-of-the-art analytical methods. The application of this cell cuture model is simple, robust, allows a long-term incubation (24 hours), and the results reveal information about the effect of eNOS activation. Thus, the relevance of this assay is obvious. Unfortunately, this assay has its limitations concerning the screening throughput, and so far all attempts to speed-up this key experiment have failed. Consequently, there exist much more published data about experiments upstream to eNOS activation. They have emphasized an enhanced transcription of the eNOS gene (promotor activity), and/or an increased level of eNOS protein, but most of them without any evidence that the eNOS enzyme is more active, and that a higher level of released NO is reached [70, 72, 85].

In order to complement eNOS activity results, further experiments as the phosphorylation at Ser^{1177} to prove activation of eNOS (section 3.1.7) and the determination of „bioavailable“ NO (DAF-FM assay; section 3.1.8) were performed. These results are consistent with the ones of the [^{14}C]L-arginine/[^{14}C]L-citrulline

conversion assay, and they offer crucial information to obtain more conclusive NO-experiments.

One could argue that primarily the measurement of the „bioavailable“ NO is required for the outcome of the isolated, active red wine constituent ethyl gallate. But the result referenced in chapter 2.6.3 illustrates that the activity guided fractionation employing the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay is essential because the DAF-FM assay has its limitations in determining reasonable results of red wine phenol extracts and fractions. For a fast detection of possibly released NO by single (isolated) compounds this assay could be an adequate option.

It is important to mention that this assay was performed to obtain results after long-term stimulation for 24 hours with both red wine and isolated constituent(s), because previous studies concerning eNOS have tested its rapid activation (short-term treatment, e.g. 15 minutes) [138], which is in contrast to the constitutively released NO level of eNOS, and does not support the argument of regular consumption of red wine to prevent endothelial dysfunction.

Employing the abovementioned assays allowed to show the positive effects of ethyl gallate concerning the eNOS system in human endothelial cells for the first time (especially the enhancement of the NO production and the consistent “bioavailable” NO result). This was possible through the systematical isolation of the eNOS activating red wine constituent ethyl gallate using activity guided fractionation.

Evidently, this simple-structured component has been identified and quantified in red wine [139, 140] as well as in other plant extracts before (e.g. walnut kernels (*Juglans regia*) [141]). Furthermore, ethyl gallate was identified as the major polyphenolic constituent in *Lagerstroemia speciosa* leaves, which are traditionally used in Asian countries for diabetes, kidney disease, as antioxidant etc. [142]. Mehla et al. isolated ethyl gallate from the galls of *Pistacia integerrima* Linn. (traditionally used in India for various inflammatory diseases) performing activity guided purification. It significantly attenuated the expression of LPS induced cell adhesion molecules (CAMs) in HUVECs

and was suggested as a lead molecule for developing therapeutic agents against inflammatory diseases [143]. The same authors found continuously out that ethyl gallate reduces acute lung injury by enhanced expression of haem oxygenase-1 (HO-1) and nuclear factor-erythroid 2-related factor 2 (Nrf(2)) activation [144]. Another gall extract (Galla Rhois) revealed EG as bioactive component inhibiting both a LPS-induced generation of NO and a higher iNOS expression in macrophages. EG increased the HO-1 expression as well as Nrf(2), which is critical for the transcriptional induction of HO-1 [145].

Ethyl gallate was also isolated from leaves of *Acacia nilotica* (L.), which is used in India as a remedy for several types of cancer. It was effective in scavenging DPPH radicals and did not possess hemolytic activity against human erythrocytes underlining its nontoxicity. Thus, EG was suggested as interesting component for the food industry [146]. Due to the fact that some gallic acid esters are listed as antioxidant food additives by the Commission of the European Communities [147] and have been used for more than fifty years, this recommendation seems to be obsolete. Consecutive investigations of *Acacia nilotica* (L.) leaves regarding acute toxicity of its main compound ethyl gallate revealed no mortality or abnormal biochemical changes *in vivo,* and moreover a protective effect on DNA and protein against oxidative stress *in vitro* [148].

The first paper mentioning ethyl gallate together with red wine performing biological activity assays to counteract the progress of atherosclerosis was published in 1999. The results of Murase et al. provided several gallic acid esters to suppress the cytokine-induced activation of Nf-κB in HUVECs highlighting the activity of EG. Nf-κB is an important transcription factor and can modulate inflammatory responses and procuagulant activities in atherosclerotic lesions. The gallates also suppressed the subsequent expression of VCAM-1, ICAM-1, and E-selectin [149].

The proliferation of VSMC provokes the development of atherosclerosis, and EG was able to inhibit serum-induced proliferation acting as single active compound as well as synergistically in combination with three other red wine components (*trans*-resveratrol, quercetin, and (+)-catechin) [150].

Australian red wines (Cabernet Sauvignon, Shiraz) were studied to identify and quantify their phenolic profiles at the different wine-making stages (samples were taken at crush, after the primary and malolactic fermentations, post-oaking, and post-bottling). The total concentration of all non-anthocyanin phenolic compounds increased during the fermentation process and reached a plateau once the wines were aged in oak barrels.

Gallic acid is the the only native hydroxybenzoic acid of *Vitis vinifera* and is present mainly in the grape seed. It was the most abundant and distinct hydroxybenzoic acid found in the wine samples of this study. The samples finally contained about 50 mg/l of gallic acid depending on the grape cultivar, geographical source and wine-making process [151]. The gallic acid contents of the referenced Australian red wines demonstrated a similar concentration compared to the selected Austrian red wine samples (gallic acid concentrations of the 60 Austrian red wines were 50.7 mg/l on average (SD = ±26.5 mg/l, n = 60); see chapter 2.1.1)

Amongst the analyzed polyphenols of these Queensland wines, there was also ethyl gallate determined during the wine-making process. The final concentration of EG after bottling was within a comparable range to the red wine samples of this thesis (50 - 65 µM; equal to a content of 10 – 13 mg/l EG and comparable to the concentration of 68 µM in the BK 05 sample; see chapter 3.1.12) [151]. Additionally, the measured concentrations of both gallic acid and ethyl gallate in Austrian red wine samples as well as in Australian red wines are confirmed by the results of Aznar et al. who performed an analysis of these constituents in ten Spanish red wines [139].

The polyphenol pattern of bottled red wine differs from the one of its grape juice and is primarily depending on the vinification process. The variety, growing area, and conditions like climate and soil influence the polyphenol pattern as well as the wine-making process [69]. Thus, a closer examination could elucidate the eNOS-activating potential of red wine constituents, which are generated during fermentation particularly focusing on the importance of ethyl gallate (section 3.1.10).

In order to quantify ethyl gallate in the mash samples a selective HPLC-method was applied (A2; chapter 2.3.1).

Furthermore, the results of our vinification experiments could be confirmed by Ginjom et al., as the EG concentration of the Australian wines developed during the fermentation process [151]. It started with very small amounts in the crush (equal to vinification sample M1), increased during primary fermentation (equal to M2 – M5), and continuously enhanced even after bottling (equal to the twofold concentration of EG in stage M6 compared to M5). The rising content of EG correlated remarkably to the increasing level of eNOS activation (see Figure 46, Figure *47*, and Figure 48).

Our explanation that ethyl gallate develops during the fermentation as a product of gallic acid in the presence of ethanol could raise question whether only the polarity of the matrix (i.e. grape juice, mash sample M1) was responsible for the detection of "extracted" ethyl gallate. Thus, a separate HPLC analysis of a pure grape seed extract was performed where ethyl gallate could not be identified (data not shown).

These results are again consistent with those of Ginjom et al., where ethyl gallate could neither be detected in the Australian Syrah sample during primary fermentation as a matter of lacking the substrate gallic acid, which is in contrast to the two Cabernet Sauvignon samples [151]. In addition, both we and Ginjom et al. found out that the EG content in red wine continued increasing after bottling and storage (sample M6, see Figure 48).

These results favor the argument of the *in situ* biosynthesis of ethyl gallate, although the abovementioned literature describes this compound occuring ab origine in other plants [141-143, 146, 148]. However, most of these articles refer to an extraction of the gallic acid ethyl ester using ethanol as solvent, thereby possibly providing conditions to develop instantly from gallic acid.

Hence, the vinification is the crucial process for the increasing concentration of the eNOS activator ethyl gallate in red wine.

Nevertheless, the presence of basically eNOS-activating agents could also be shown in the non-fermented grape juice (mash sample M1). These additionally activating

compound(s) besides ethyl gallate are probably more polar one(s). The eNOS activity results of assaying the apolar fractions (AF) and polar fractions (PF), which were obtained by liquid-liquid partition (see section 3.1.2.1), supported this finding. Each AF of the four samples indicated an enrichment of the eNOS activating constituent(s), and one PF (sample PW 04, see Figure 18) demonstrated eNOS activation. Furthermore, the effect of the PF of BWG 07 sample (section 3.1.4.1), which was finally exerted to isolate ethyl gallate from the AF, was consistent with the PF of PW 04. These data prove that there are several red wine compounds of different polarity influencing the eNOS activity (see Figure 18 and Figure 25). PF contains particularly polar anthocyanins, of which several authors have suggested a positive effect on eNOS (e.g. cyanidin-3-glucoside [152], malvidin-3-glucoside [153]).

The majority of red wine studies describe malvidin-3-glucoside as the main monomeric anthocyanin with amounts of up to 200 mg/l during fermentation and 90 mg/l after bottling (equal to a concentration of 180 µM) [151, 154]. Malvidin-3-glucoside suppresses pro-inflammatory mediator NfKB, up-regulates eNOS mRNA and enzyme phosphorylation after 6 hour stimulation at 25 µM in BAECs, suggesting enhancement of eNOS activity and NO production and therefore benefits cardiovascular health [153]. Cyanidin-3-glucoside induces eNOS protein expression in a dose- and time-dependent manner and increases also the nitric oxide output after long-term incubation of bovine artery endothelial cells (BAECs) [155].

Another publication compared the activity of cyanidin-3-glucoside, its degradation product protocatechuic acid and phase II metabolite, vanillic acid using established human vascular cell models. Nitric oxide levels were not modulated by the treatments, although eNOS was upregulated by cyanidin-3-glucoside, and superoxide production was decreased by both phenolic acids. Vanillic acid did partially influence NADPH oxidase and haem oxygenase-1. Anthocyanin metabolites are therefore proposed to modulate vascular reactivity by inducing HO-1 and modulating NADPH oxidase activity, resulting in reduced superoxide production and improved NO bioavailability [156].

The more lipophilic anthocyanin derivative Petunidin 3-O-(6''-p-coumaroyl-glucoside) was identified as potent eNOS activator tested at 100 µg/ml. Auger et al. aimed to isolate red wine phenolic compounds capable of activating eNOS in endothelial cells using multi-step fractionation. The short-term (15 min) phosphorylation level of Akt and eNOS was determined by Western blot analysis. The active fractions and subfractions were generated using size-exclusion chromatography and semi-preparative RP-HPLC, respectively, and contained mainly procyanidins and anthocyanins [138].

The result could be an explanation for the eNOS activation of the PF of sample PW 04 and BWG 07, where we assumed the effect to be attributed to constituents such as anthocyanins.

Wallerath et al. assumed that only the total amount of polyphenols was relevant for the activation of the eNOS enzyme [70]. Another study analyzed the antioxidant capacity and polyphenols of wines from diverse varieties and vintages from Southern France. The obtained results suggested that depending on the antioxidant activity the total content of phenolic compounds could be more important than the content of the individual components. The different wine-making techniques were reported to be important factors influencing polyphenolic quantities in wine [157].

In the early stages of our activity guided fractionation we obtained a red wine concentrate and two fractions (DRWC, FE, and RWPE; see chapter 3.1.1.3), which showed differences in eNOS activation. The increased enzyme activity of eight DRWCs correlated to the total phenol contents of these red wines (see Table 18 and Figure 13) suggesting the quantitative amount of polyphenols to be important for an increased eNOS activity. But, stimulation with the defined quantity of RWPE (600 µg/ml) revealed differences in the eNOS activation pattern, indicating that the polyphenolic fingerprint is crucial for the enhanced activity (Figure 16).

Previous studies have demonstrated an effect on the eNOS system (promotor activity, eNOS expression, eNOS activity and bioavailable NO level) after long-term incubation with red wine phenolic extracts of several varieties (Merlot, Pinot Noir, Tempranillo,

Zinfandel, Trollinger, etc.) from various origins (France, Italy, Germany, Chile, Australia, etc.), and support the present findings regarding both the quantity of relevant phenols and the composition of RWP [69, 71, 72]. Raethel et al. emphasized the influence of variety, growing area, and conditions like climate and soil [69].

Moreover, we investigated the storage conditions as part of the wine-making process, which could have additionally contributed to the polyphenolic fingerprint (see chapter 3.1.13). Previous examinations revealed that the storage of red wine in an oak barrel did not positively influence eNOS promoter activity [158]. In our case the influence on eNOS activity of red wine storaged in a steel cask compared to a barrique cask was of interest.

The maturation process using barrique casks did not alter eNOS activation ability compared to steel casks. Thus, the presence of ellagitannins in this red wine could not demonstrate an additional positive impact on eNOS activation.

In order to detect a possible effect on the eNOS enzyme activation as well in white wine a typical Austrian sample (GV; “Grüner Veltliner”) was determined (chapter 3.1.11). Lack of activity is obviously related to both the qualitative and the quantitative difference of polyphenols. We presumed this white wine sample not to include ethyl gallate as constituent, which could be confirmed comparing an HPLC chromatogram with several red wine samples (Figure 52). A possible explanation for this discrepancy of eNOS activating ethyl gallate concentration could be the difference of the red and white wine-making process (personal communication with Dr. R. Eder, 2010). As a matter of taste white wine is fermented after the immediate separation of mash, and is therefore lacking the substrate gallic acid for biosynthesis of ethyl gallate during vinification. In contrast, red wine fermentation is based on several weeks of maceration.

This fact was supported by Sparwel et al., who reported catechins – present in red wine but not in white wine – to inhibit the PDGF receptor. The authors produced a white wine the same way as usually exerted for red wine and obtained the same biological activity as previously found in red wine [23]. Another study, measuring the

antioxidant activity of polyphenols and wine, provided the proof-of-principle. Dry white wines usually show the lowest antioxidant activity correlating with both the lowest content of total polyphenols as well as the lowest concentration of gallic acid. Hence, a Chardonnay was made using the identical technique performed for red wine-making succeeding in enrichment of phenols. Therefore, the antioxidant capacity level of this wine and its content of gallic acid (25 mg/l) reached the level of some red wines [157].

These results confirm our presumption that experiments processing white wine the similar way as red wine would reveal eNOS activity enhancement by spontaneously developed ethyl gallate during fermentation.

Yet, these white wine data raised question about the outcome of Eder et al. [159], who presented some diverging results, as Styrian white wines induced eNOS activity enhancement. A possible explanation might be ascorbic acid, which is sometimes added up to 150 mg/l to Austrian white wines as food additive (E 300) to avoid oxidation ("browning-effect" occurs principally in white and rosé wines due to oxidation) [160]. We used ascorbic acid as positive control for the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay at a concentration of 100 µM. When we compare this to a possible concentration of ascorbic acid up to 1 mM in white wine one could argue that this compound is even responsible for the eNOS activity enhancement and thus provoking a wrong positive result.

The dietary flavanol (-)-epicatechin is a major active constituent of beverages such as cocoa drinks, tea and red wine and also of flavanol-rich foods. It has been shown to improve endothelial function inducing relaxation of the endothelium, mainly by eNOS activation. Epicatechin counteracts hypertension and endothelial dysfunction in animal models and humans [161]. An *in vivo* study in healthy men suggested (-)-epicatechin to activate the eNOS system to augment the nitric oxide status and may thereby improve endothelial function, based on acutely increased plasma and urine nitrite levels [162]. Persson et al. studied the influence of tea flavanols on the endothelial nitric oxide synthase and demonstrated that (-)-epicatechin raised the nitric oxide level in HUVECs

after incubation for 24 hours [163]. In this context, our data did not demonstrate increased eNOS activity. The semi-preparative fractionation of the AF of BWG 07 using HPLC (see chapter 3.1.4.2) showed significant improvement of NO production only in subfraction F3.3.1, representing isolated ethyl gallate.

The red wine constituent (-)-epicatechin was tested beside other polyphenols (catechin, *trans*-resveratrol) at naturally occurring concentrations for the potential to inhibit endothelin-1 (ET-1 is the physiological counterpart of the nitric oxide synthase) in order to study the direct impact on the vascular wall of isolated rat aortic rings. Only (-)-epicatechin was able to decrease the endothelial release of the vasoconstrictor ET-1 *in vitro*, and thus prevented endothelial dysfunction and consequently improved endothelial function [161]. This observation was in accordance with Corder et al., who investigated the relation between oligomeric proanthocyanidins (OPC) content of a large number of red wines and the increased longevity of the population of Mediterranean countries. The *in vitro* inhibition of ET-1 was coupled with the degree of OPC oligomerization attributing more impact to the higher polymerized molecules [164].

A previous work has shown that a grape seed extract caused endothelium-dependent relaxation. The bioassay guided fractionation using rat aorta for *ex vivo* experiments exhibited proanthocyanidin trimers, tetramers, pentamers, and polymers and their gallates, as well as a dimer gallate as the most active components (EC50 values in the range of 0.6 – 2.5 µg catechin equivalents/mL). These (-)-epicatechin oligomers were identified applying HPLC retention times and electrospray-ion-trap mass spectrometry [165].

The MS/MS data of the isolated eNOS-activating red wine constituent in this thesis revealed the presence of a gallic acid fragment conjugated to an ethyl moiety, which was linked rather to the carboxyl function than to one of the three phenolic hydroxyl groups. After confirmation by UV-spectrum and HPLC retention time the eNOS-activating constituent could be identified as ethyl gallate (EG; gallic acid ethyl ester).

We presumed that EG could be a marker substance for eNOS activity of red wine, and therefore the EG concentrations were correlated with eNOS activity levels of the DRWCs (see Figure 53). Seven red wine samples were analyzed together with the white wine sample for the content of EG in pure wine. The amounts of EG in these red wines varied from about 4 mg/l to 14 mg/l (i.e. concentrations from 20 µM to 70 µM). These tested red wine samples showed a good correlation between the ethyl gallate concentrations and the increased eNOS activity (Figure 53). At least some of the samples deviate in their eNOS activation, which could be explained by additionally influencing red wine constituents. Although ethyl gallate cannot be exclusively responsible for the increased activity, it should nevertheless be regarded as an important marker for *in vitro* eNOS activity enhancement in red wine preventing endothelial dysfunction.

Gallic acid was assayed at the similar concentrations as EG and interestingly did not alter eNOS activity at all. In this context, the findings suggest that the esterification by the ethyl group is critically necessary for the functionalization of gallic acid to facilitate eNOS activation.

A closer look at the HPLC chromatogram revealed noticeable similarities between ethyl gallate and another identified gallic acid derivative, namely syringic acid (gallic acid 3,5-dimethylether). Those similarities lie in the retention time, the identical MW of 198 Da, a matching UV-spectrum, and the same precursor molecule (gallic acid) (see chapter 3.1.5). However, our data do not confirm the results of Simoncini et al. claiming an effect of syringic acid (besides other polyphenols) on the eNOS enzyme activation after long-term stimulation [166].

To underpin our research further gallates (methyl (MG), propyl (PG), butyl (BG), isopentyl (IG), octyl (OG), and lauryl (LG) gallate) were equally tested. The structure-activity-relationship revealed a higher eNOS activity after treatment with PG, BG, and IG compared to EG, even if stimulated at lower concentrations, whereas OG and LG showed a decrease of eNOS activity. The latter could be explained by the solubility threshold of these lipophilic compounds in aequous matrix and/or too high

concentrations for endothelial cell experiments. Thus, the influence of these two compounds should be investigated in further studies to analyze a possible effect at lower concentrations than assayed and presented in chapter 3.1.6.

To date, the effects of gallates have been subject to various studies concerning antioxidant activity [167], inhibition of *Chlamydia pneumoniae* infection [128], anticancer properties [168], influence on 5 alpha-reductase [169], and antibiotic potential [170]. As all of these alkyl esters of gallic acid show scavenging activity towards reactive oxygen species depending on the polyphenolic structure, some of them are listed as antioxidant food additives by the Commission of the European Communities. These are propyl gallate (E310), octyl gallate (E311), and lauryl gallate (E312) preventing especially fat and oil containing food products, chewing gum, and snacks from oxidation to prolong shelf-life with an ADI of 0.5mg/kg [147].

Another well-known red wine compound, which demonstrated vascular bioactivity, is the flavonol quercetin. It is one of the most abundant flavonoids present in plants and in food, such as apples, onions, and red wine [171]. Amongst some other red wine polyphenols, quercetin is held responsible for the beneficial effects of the “French Paradox”. Thus, a large number of studies investigated its effects on the modulation of the endothelial system.

The application of quercetin has demonstrated an improvement of endothelial dysfunction by increasing NO synthesis in HUVECs enhancing the intracellular Ca^{2+} concentration [172]. Several studies using isolated rat aorta for their experiments reported quercetin to induce endothelium-dependent vasorelaxation, to enhance NO production and increase the bioavailability of NO. Moreover, it exerts direct vasodilator effects on the blood vessel by nitric oxide-mediated mechanisms and prevents both AngII-induced endothelial dysfunction and ET-1-induced NADPH oxidase up-regulation leading to endothelial dysfunction [57, 173-175]. Further studies employed *in vivo* animal models. One has shown that quercetin reduces the increase in blood pressure and heart rate and enhances the endothelium-dependent aortic vasodilation in sponataneously hypertensive rats [176]. Another one pointed out that

quercetin inhibits the development of L-NAME-induced hypertension dose-dependently, but this effect could not be observed in normotensive animals [177]. In healthy humans, quercetin augments the nitric oxide status and reduces endothelin-1 concentration and could thereby improve endothelial function [162].
Further flavonoids, such as myricetin and isorhamnetin, were able to activate eNOS via phosphorylation in HUVECs and to inhibit induced eNOS deficiency to prevent endothelial dysfunction, respectively [174, 175, 178].

There exists remarkable interest in elucidating positive effects of polyphenols counteracting endothelial dysfunction, and cell culture experiments are the most common methods to obtain results. Quercetin, however, is often being put into question (like some other polyphenols, e.g. gallic acid, anthocyanins etc.) because it is a redox active compound and could initiate an immediate generation of hydrogen peroxide in cell culture medium, possibly influencing the outcome of a study.
Several cell cultural media (Dulbecco's modified Eagle medium (DMEM), McCoy's 5A medium, and RPMI 1640 medium) were tested for the development of H_2O_2 levels after addition of quercetin, gallic acid, catechin, epigallocatechin gallate and epigallocatechin. All the examined polyphenols promoted a significant increase of hydrogen peroxide concentration in these cell culture media. This effect was considered to manipulate reported results [179]. Quercetin showed a reduced stability in cell culture medium growing human colon carcinoma cells (HT29) and simultaneously provoked the generation of hydrogen peroxide. The presence of either catalase or ascorbic acid improved its stability *in vitro* [180]. A recent study characterized and compared the stability of 53 antioxidants (flavonols, flavones, isoflavones, catechins and stilbenoids) incubated in DMEM at 37 °C. With regard to the degree of hydroxylation, glucosidation and methylation there was an obvious impact on the stability of the polyphenols. But the compounds were clearly more stable in human plasma than in DMEM. Consequently, the authors recommended applying stability tests in parallel with cell culture experiments for dietary antioxidants in order to avoid artifacts [181]. Kern et al. employed HPLC/DAD analysis as well as the

measurement of growth inhibition of HT29 colon carcinoma cells growth inhibition in order to investigate the stability of anthocyanidins and their degradation products, which are simple hydroxybenzoic acids, in DMEM. Only gallic acid, which originated from delphinidin, demonstrated growth inhibitory properties. However, under cell culture conditions delphinidin was only detectable for 30 minutes. Gallic acid reached only a maximum of 20 % of the applied delphinidin concentration after one hour indicating chemical instability too. Moreover both compounds induced a substantial formation of hydrogen peroxide, which could be antagonized by adding the enzyme catalase. Therefore, their presence in the medium was prolonged and the growth inhibitory properties of both were positively affected [135].

On the one hand the modulation of endothelial cell functions could be negatively influenced by hydrogen peroxide concentrations (e.g. tetrahydrobiopterin deficiency, uncoupling of eNOS) and on the other hand the eNOS expression could be upregulated *in vitro* and in animal models of atherosclerosis [182].

To summarize, all these references lead us to the conclusion that results, especially from *in vitro* assays using cell culture media to test polyphenols for biological activity, have to be interpreted carefully. The fact that polyphenolic structures show a detectable reactivity (e.g. antioxidants) implicates the instability of these molecules under certain conditions. The unintentional development of hydrogen peroxide may contribute to the generation of artifacts, and subsequent results may therefore be based on unknown errors.

Hence, we confirmed our eNOS activity data gathered from [14C]L-arginine/[14C]L-citrulline conversion assay by additionally performing an adapted assay (catalase assay, see chapter 2.5.3) to suppress hydrogen peroxide. An influence of *in vitro* generated H_2O_2 on both the EG- and the RWPE-induced eNOS activation was not detectable (compare Figure 25, Figure 37 and Figure 42). Moreover, the result of gallic acid, which is one of the polyphenols referenced to promote hydrogen peroxide generation [135, 179], did not alter eNOS activity compared to the control level performing the [14C]L-arginine/[14C]L-citrulline conversion assay (see Figure 39). Therefore, a conceivable

presence of hydrogen peroxide in the used cell culture medium did not seem to affect the measured eNOS activity enhancement.

Based on the outcome of a predecessor's studies, the objective of this thesis was to identify eNOS-activating red wine components via activity guided fractionation because assaying *trans*-resveratrol could not explain the enhanced NO level observed with red wine polyphenol extract (RWPE) after long-term incubation. The previously calculated concentration of 600 µg/ml of RWPE to test for modulations of the eNOS enzyme was related to its *trans*-resveratrol content, which should reach "comparable to red wine" concentrations in the cell culture medium (1 µM – 10 µM) [69]. Our analysis of the *trans*-resveratrol content of the 60 Austrian red wine samples revealed 1.49 mg/l (SD = ±1.02 mg/l) on average equal to 6.5 µM (SD = ±4.4 µM) (see chapter 2.1.1). The red wine sample containing the highest amount of *trans*-resveratrol was a Blaufränkisch 2005 from the region Mittelburgenland (4.48 mg/l, equal to 19.7 µM), which was among the eight selected red wine samples to measure eNOS activation (see section 3.1.1), but sorted out after the First Test Series. In addition, the EG concentration of this sample was 6.4 mg/l (equals 32 µM, see Figure 52, chapter 3.1.12). Concerning the latter mentioned sample, only a minor effect of *trans*-resveratrol on eNOS activity could be revealed as a result of the First Test Series (see section 3.1.1.3), although containing ethyl gallate, however. Besides, fraction F4 – a subfraction of the AF of sample BWG 07 to isolate ethyl gallate – represents the complex pattern of red wine flavonoids and stilbenes, and showed a slightly significant eNOS activation. This effect could be assumed to derive either from *trans*-resveratrol or quercetin. Figure 19 and Figure 23 demonstrate the longer retained compounds of AF in the respective chromatograms. The figures in chapter 3.1.4.2 illustrate the semipreparatively obtained fraction F4 and the related effect on eNOS activation.
Several authors assayed for eNOS activity treating EA.hy926 endothelial cells with *trans*-resveratrol. Concentrations for a significant long-term effect were ranged between 20 µM and 30 µM [84, 86, 183]. Appeldoorn et al. also discussed the physiological relevance of such *in vitro* results, when a treatment above 30 µM is

necessary to demonstrate influence [183], unlike physiological concentrations of polyphenols in humans are generally below 10 µM [171]. Schmitt et al. investigated the contingency of a synergistic effect between *trans*-resveratrol and hydroxytyrosol [86]. Another publication reported trans-resveratrol to increase the bioavailabilty of NO significantly at 30 µM in HUVECs. The treatment did not influence eNOS activity *in vitro*, suggesting that *trans*-resveratrol had no direct effect on eNOS, but acts via mobilization of Ca^{2+} [184]. Ladurner et al. studied the relevance of major resveratrol metabolites, which are particularly sulfate and glucuronide conjugates, on the eNOS enzyme activity. Moreover, the authors noted that the relevance of *in vitro* data for the interpretation of *in vivo* effects of resveratrol is controversial [84]. Biovailability of unmetabolized resveratrol *in vivo* is rather limited although 70 % of the orally administered amounts are rapidly absorbed. Only traces (< 5 ng/ml) were detectable in plasma after oral dosage of 25 mg whereas the total concentration of metabolites was measured in a micromolecular range (2 µM) [83]. Observations to study chemopreventive activity revealed plasma concentrations of resveratrol at 2.4 µM after an intake of a 5 g single oral dose, and 3- to 8-fold higher concentrations of the sulfate and glucuronide metabolites, respectively [185].

However, to propose the consumption of red wine for health properties requires studies of the bioavailability and subsequently of detectable metabolites. There exist a large number of *in vitro* data demonstrating several effects of compounds or phenolic substance classes, but translation of such results to highly relevant *in vivo* data would be the inevitable task [186]. Stockley et al. concluded that the concentrations of polyphenolic constituents, which were used to obtain the majority of *in vitro* data, were not compellingly in accordance with those observed in wine [186].

Hence, the quantification of the eNOS-activating red wine compound ethyl gallate indicated that the concentrations used to assay for modulation of eNOS enzyme correlated to the detected amounts in Austrian red wines. This findings were additionally confirmed by the EG concentrations found in Spanish and Australian red wines [139, 151].

Interestingly, the ethyl gallate precursor gallic acid is an extremely well absorbed compound compared to other polyphenols (e.g. proanthocyanidins). The maximum concentration of gallic acid in plasma including its major metabolite 4-O-methylgallic acid [187] was 4 µM after ingestion of 50 mg pure gallic acid [171], and both appeared rapidly in plasma and urine [188]. Such an intake seems plausible because this quantity is not unlikely to be ingested when consuming red wine. Generally, there exist not much data about absorption and metabolism of hydroxybenzoic acids, such as gallic acid [189, 190]. But a good absorption is not explicitly related to galloylation as epigallocatechin gallate showed reduced absorption compared to epigallocatechin [171].

Concerning ethyl gallate, an *in vitro* dissolution study employed gastric and intestinal fluids, and the ethyl gallate concentration in the wine samples was stable after gastric and intestinal treatment. The ester structure was held responsible tor the stability of ethyl gallate [191]. Another publication examined the plasma levels of ethyl gallate and its metabolites after intragastric administration of EG to rats, performing LC-MS/MS experiments. The pharmacokinetics of EG revealed a fast metabolism within one hour generating three metabolites, of which the first was identified as gallic acid, the second was a sulfated EG, and the third one was additionally assumed to be a methylated homologue. Ratios and concentrations of the metabolites compared to ethyl gallate have not been analyzed in this study [142].

Summarizing, these data about ethyl gallat improve the understanding of red wine's benefical effects and represent the foundation for further investigations.

4.2 Red wine and *Chlamydia pneumoniae*

Atherosclerosis represents a complex disease which cannot be explained by one single cause. Besides classical risk factors for cardiovascular diseases, new ones such as microbial infection have been reported [192]. It could be demonstrated *in vitro* and *in vivo* that *Chlamydia pneumoniae* provokes atherosclerosis by multiple mechanisms including inflammation, amongst others induced by inflammatory cytokines, and dysregulation of important cellular functions of the vasculature, such as NO synthesis [193, 194].

Treatment with numerous antibiotics could counteract chlamydial infection together with atherosclerosis, but results showed a subsequent reversion of both diseases after a few months [195].

The intake of naturally occurring polyphenols against chronic diseases is subject of several studies [196-198]. Hence, red wine is related to beneficial health effects in this context due to the high content and complex constitution of polyphenols.

The correlation between red wine, *C. pneumoniae*, and atherosclerosis has been studied to date only once addressing the antichlamydial effects of pure red wine as well as the main component *trans*-resveratrol. As the activity of plant extracts is hardly ever based on a single compound the antichlamydial activity in case of red wine is most likely dependent on multiple compounds [120, 126, 199].

To find out further polyphenols inhibiting *C. pneumoniae* infection a closer examination of the red wine composition was performed using activity guided fractionation.

For this purpose, the fractions and isolated components of the red wine samples (BK 05 and ZC 05) which were used for eNOS activity investigations (see sections 3.1.1 – 3.1.3) were applied. Initial fractionation showed inhibition of *C. pneumoniae* growth, and the consecutive sub-fractions of one red wine (BK) revealed the isolated compound ethyl gallate, predominantely responsible for antichlamydial effects. EG was considerably inhibiting inclusions and growth of *C. pneumoniae*, and thus was the most potent one of the identified compounds.

The potential of EG against chlamydial inclusions was determined dose-dependently, and the MIC of the tested concentrations was detected at 50 µM (Figure 59).
The effective quantity of EG against *in vitro* chlamydial infection is interestingly within a similar range like eNOS activity analyses (chapter 3.1), and underlines the presence of an adequate concentration of EG even in the pure red wine sample (BK).
These findings present several red wine components with a modest activity against *C pneumoniae,* which is in accordance to previous investigations for antichlamydial polyphenols [120]. Some well-known red wine constituents, such as *trans*- and *cis*-resveratrol, primarily inhibited the formation of chlamydial inclusions of the CWL-029 strain between 50 µM and 100 µM concentration (data not shown), corresponding to the chlamydial growth inhibition of these two isomers [120]. This antichlamydial activity of resveratrol strikes different *C. pneumoniae* strains [126], but such concentrations of *trans*- and *cis*-resveratrol could definitely not be analyzed in pure red wine (concentrations of the selection of 60 Austrian red wines of *trans*-resveratrol and *cis*-resveratrol on average were 6.5 µM (SD = ±4.4 µM, n = 60) and 4.5 µM (SD = ±2.4 µM, n = 60), respectively; see chapter 2.1.1).

Antichlamydial activity of EG is comparable to the antichlamydial effect of the gallic acid ester octyl gallate *in vitro* [120]. Such gallic acid esters are hydrolyzable to gallic acid, what could already be demonstrated by performing *in vivo* experiments with octyl gallate [128, 200]. Therefore, the *in vitro* effect of ethyl gallate concerning *C. pneumoniae* has to be confirmed in adequate animal models. Moreover, it should be investigated, whether *in vivo* concentration of EG reaches relevant levels for antichlamydial activity. As the toxicity of gallic acid is rather low [201], it could be applied in quite high amounts. But esters, such as EG, which are more efficient antichlamydial compounds, should be subject to more detailed investigations.

4.3 Conclusion

Endothelial dysfunction is a hallmark in the development of atherosclerosis. Both the improvement of the endothelial nitric oxide production and bioavailability as well as the inhibition of pro-atherosclerotic *Chlamydia pneumoniae* infection play a crucial role for vascular functionality.

In conclusion, the results of this study prove that the systematic activity guided fractionation is an adequate and important approach to profile for active components in red wine. The employed assays were appropriate models to obtain significant and conclusive results elucidating some modes of action of ethyl gallate. This compound represents a tool providing a better understanding of eNOS enzyme regulation and *Chlamydia pneumoniae* inhibition. The effectiveness of red wine is not completely explainable by one single compound neither by ethyl gallate nor by *trans*-resveratrol. But the requested improvement of NO production could be allocated to the isolated and identified ethyl gallate in this *in vitro* study for the first time. Our working hypothesis was that *trans*-resveratrol cannot be the exclusively responsible red wine constituent to prevent endothelial dysfunction because it was already proved that it only enhances the first part of the eNOS cascade until increased expression of the eNOS protein at usual concentrations in red wine (see Figure 6). Ethyl gallate is significantly influencing eNOS activity and NO release, and therefore completes the second half of the eNOS cascade. Possibly, a synergistic effect of these two compounds on eNOS regulation could be observed performing further studies.

Ethyl gallate is demonstrating its multipotential character, because together with the mentioned effects on eNOS it was additionally identified as remarkable antimicrobial agent indicating inhibitory effects against *Chlamydia pneumoniae*. Notably, for the functionalization of gallic acid the esterification to ethanol is necessary, thereby obtaining ethyl gallate. The basic principle is the wine-making technique especially applied for red wine submitting the substrate gallic acid to the generated ethanol gradient due to fermentation. The ethyl gallate concentration moreover increases during storage.

Besides employing various chromatographic techniques to guide the fractionation and isolation steps of ethyl gallate a separate HPLC-method was developed to perform a fast, simple and selective analysis directly in red wine. This was additionally correlated to the eNOS activation of some red wine samples. Although red wine represents a high complexity of polyphenols and obviously contains further contributing phenolic compounds, ethyl gallate is the outstanding constituent indicating enhancement of NO production and release, and therefore has to be regarded as biomarker for *in vitro* eNOS activation. Remarkably, the measured contents of ethyl gallate in Austrian red wines are even supported by previous results of Australian and Spanish samples. These were consistent with the effective *in vitro* concentrations of ethyl gallate for all applied assays.

This is the first time ethyl gallate is described to show effects counteracting both endothelial dysfunction and *Chlamydia pneumonia*e infection. Thus, our work represents a gain of basic knowledge giving valuable leads for prospective experiments. For the moment, there is a lack of bioactivity studies (especially artery models and *in vivo* studies) attributed to ethyl gallate to interpret and compare bioavailability, metabolism and metabolites affecting the activity. Hence, complementary investigations ought to be exerted continuing elucidation of this red wine constituent.

Altogether, ethyl gallate contributes to the understanding of both eNOS regulation as well as inhibition of *Chlamydia pneumoniae* infection. Concluding, it provides a potential dual long-term effect preventing the formation of atherosclerosis and has to be considered a relevant in vitro contribution to explain beneficial effects of moderate and regular consumption of red wine, well-understood as “French Paradox”.

5 SUMMARY

Since the early nineties the term “French Paradox” has been used to describe the discrepancy between the lower-than-expected mortality rate of cardiovascular diseases (CVDs) and a diet rich in saturated animal fat in certain areas of France. For this beneficial, cardio-protective effect a regular and moderate consumption of red wine is supposed to be responsible. Atherosclerosis is associated with CVDs as a result of a critical pathophysiological process in the vascular system, termed endothelial dysfunction. Clinical and experimental data have proven that the production of nitric oxide (NO) by the endothelial nitric oxide synthase (eNOS) is an essential factor in the cardiovascular system to sustain a healthy endothelium and vascular homeostasis. Therefore, maintenance or even improvement of eNOS activity is required in order to antagonize the development and progress of atherosclerosis.

Previous *in vitro* studies with red wine phenol extract (RWPE) have illustrated remarkably that red wine is able to beneficially influence the eNOS system after long-term incubation for 24 hours [71]. Based on these results, further experiments with the most prominent red wine compound *trans*-resveratrol, as possible initiator of such effects, were executed. Therefore relevant concentrations, which can also be found in red wine (1 µM and 10 µM), were tested. Yet an effect could only be revealed on eNOS promotor activity, on stabilization of mRNA and on eNOS enzyme expression, but neither on eNOS activity itself nor on released NO. Consequently, the impact of previously investigated RWPE on eNOS cannot be attributed exclusively to *trans*-resveratrol.

Hence, the first objective of this thesis was the activity-guided isolation and identification of polyphenolic red wine constituent(s), particularly responsible for the enhancement of eNOS enzyme activity, by performing the [14C]L-arginine/[14C]L-citrulline conversion assay. Additionally, the detection of phosphorylation of eNOS regulatory domain Ser^{1177} and the measured release of bioavailable NO by employing

the 3-Amino, 4-aminomethyl-2',7'-difluorofluorescein assay should underline the eNOS activity experiments.

Atherosclerosis represents a complex disease and its prevalence cannot be related to one single cause. Besides classical risk factors for cardiovascular diseases, new risk factors such as microbial infections have been reported. *Chlamydia pneumoniae*, a Gram-negative bacterium, is well-known to cause infections of the respiratory tract, but over the past years several studies have also pointed out its contribution to atherosclerosis [97, 104]. Thus, the second part of this work concentrates on the identification of new antichlamydial hit(s) to corroborate that an explanation for the French Paradox implicates various aspects. Therefore, the identification of such red wine constituents may provide new strategies to face atherosclerosis and CVDs.

To systematically approach the aim of active substance identification, eight Austrian red wine samples (2x Blaufränkisch, 1x Zweigelt, 2x St. Laurent, 1x Pinot Noir, 1x Lagrein, 1x Merlot) were selected out of 60 representative red wines due to their very high, respectively very low content of main polyphenolic constituents. Dealcoholized red wine concentrates (DRWC) of the samples were tested together with the obtained fractions FE (First Eluate) and RWPE (Red Wine Phenol Extract) for eNOS activity enhancement. Endothelial cells showed differences in the increase of eNOS activity after treatment with the tenfold concentrated DRWCs, which correlated to the total phenol contents of the eight red wine samples suggesting the quantitative amount of polyphenols to be important for an increased eNOS activity. However, the stimulation with the defined quantity of RWPE (600 µg/ml) revealed differences in the eNOS activity pattern, indicating that the polyphenolic fingerprint is crucial for the enhanced activity as well. Thus, the continued multi-step fractionation of RWPEs of the four most eNOS-activating red wine samples generated Apolar Fractions (AF) and Polar Fractions (PF) by liquid-liquid partition using ethyl acetate and water. All AFs exposed an enrichment of eNOS activating constituent(s), and consequently the focus for further fractionation was on AF of the most potent red wine sample

(Blaufränkisch 2005, Klosterneuburg). The performance of solid phase fractionation with C18-cartridges and a stepwise MeOH-gradient resulted in five solid phase fractions (SPFs), of which only SPF 3 achieved eNOS activation similar to the related AF. An HPLC-analysis of the five generated fractions displayed one outstanding peak. To effectively elucidate the structure of this component the fractionation was restarted with another red wine sample (Burgweingartl 2007, Klosterneuburg). The fractionation approach was modified, and instead of the SPE, a semi preparative HPLC-method (RP-18e) was employed. The three subsequently executed activity-guided fractionation steps resulted in the isolation of the eNOS-activating compound, which was identified by applying an HPLC/MS analysis. MS/MS data indicated the presence of a gallic acid fragment conjugated to an ethyl moiety, linked to the carboxyl function, which could be identified as ethyl gallate. These findings suggest that the esterification is critically necessary for the functionalization of gallic acid to facilitate eNOS activation.

The influence of ethyl gallate on eNOS activity in human endothelial cells after long-term treatment is now described for the first time (between 10 µM and 100 µM). Moreover, complementary experiments (bioavailability of released NO and the increase of phosphorylation at Ser^{1177}) underline the effect of ethyl gallate on the eNOS-system. These results are supported by eNOS activation of further gallic acid esters.

It could also be proved that the red wine vinification process is relevant for the development of eNOS-activating red wine constituents. The activating potential of compounds in non-fermented grape juice is marginal. Subsequently, a selective HPLC-analysis detected ethyl gallate to be the main responsible constituent with 20 to 70 µM, which develops in red wine during fermentation. Furthermore it could be demonstrated that the effect of ethyl gallate on eNOS activity increases along with the duration of the vinification process. The same analysis was repeated with a white wine sample, in which ethyl gallate could not be found.

Finally, the influence of red wine storage on eNOS activation was investigated by comparing steel cask to barrique cask storage. After 6 months no difference could be observed. Thus ellagitannins could be unveiled as irrelevant compounds.

The second part was the activity-guided fractionation of red wine to find new polyphenolic inhibitors of *Chlamydia pneumoniae*. The fractionation was started analogously to the above mentioned scheme using two red wine samples (1x Blaufränkisch, 1x Zweigelt). The most active fraction (AF of Blaufränkisch 2005, Klosterneuburg) was further separated by semi-preparative HPLC, and ten subfractions were obtained. After identification of several red wine constituents both the fractions and the corresponding pure compounds were assayed *in vitro* for their potential to inhibit *C. pneumoniae*. While various fractions and pure compounds are moderately able to inhibit *C. pneumoniae,* one specific fraction containing ethyl gallate, displayed outstanding anti-chlamydial effects, not only in growth inhibition but also in the reduction of *C. pneumoniae* inclusions. The potential of ethyl gallate against chlamydial inclusions was determined dose-dependently, detecting MIC at 50 µM.

To conclude it can be stated, that through the underlying analysis of this thesis ethyl gallate could be isolated and identified from red wine for the first time, using activity-guided fractionation. Its multi-potent character was verified *in vitro*, as the positive influence on the eNOS system and the antichlamydial effect could be proved.

6 ZUSAMMENFASSUNG

Seit den frühen Neunziger Jahren wird der Begriff „Französisches Paradoxon“ herangezogen, um die Diskrepanz zwischen der ungewöhnlich niedrigen Mortalität durch Herzkreislauf-Erkrankungen und einer Ernährung reich an tierischen, gesättigten Fetten, die in manchen Regionen Frankreichs typisch ist, zu erklären. Ein regelmäßiger, moderater Rotweinkonsum wird für diesen positiven, kardioprotektiven Effekt verantwortlich gemacht. Aufgrund der sogenannten endothelialen Dysfunktion, einem kritischen pathophysiologischen Prozess im Gefäßsystem, wird Atherosklerose in engem Zusammenhang mit Herzkreislauferkrankungen betrachtet. Klinische und experimentelle Studien belegen, dass die Produktion von Stickstoffmonoxid durch die endotheliale Stickstoffmonoxidsynthase ein entscheidender Faktor für die Homöostase ist. Um der Entstehung und dem Fortschreiten der Atherosklerose entgegenzuwirken ist es also notwendig, die eNOS Aktivität aufrecht zu erhalten oder sogar zu erhöhen.

Vorangegangene *in vitro* Studien mit Rotwein Phenol Extrakt (RWPE) mit einer Stimulationsdauer von 24 Stunden haben auf beeindruckende Weise gezeigt, dass Rotwein das eNOS-System positiv beeinflusst. Ausgehend von diesen Ergebnissen wurden weitere Experimente mit dem bekanntesten Rotwein-Inhaltsstoff trans-Resveratrol, als möglichem Initiator solcher Wirkungen, durchgeführt. Dafür wurden relevante Konzentrationen so wie sie in Rotwein vorkommen können getestet (1 µM and 10 µM). Dabei konnten lediglich Wirkungen auf die eNOS Promotor-Aktivität, auf die Stabilisierung von mRNA und auf die eNOS Enzymexpression nachgewiesen werden, jedoch weder auf die eNOS Aktivität selbst noch auf das freigesetzte NO.
Folglich lässt sich die Auswirkung des bereits untersuchten RWPE auf die eNOS nicht ausschließlich auf trans-Resveratrol zurückführen.

Daher war es das primäre Ziel der vorliegenden Arbeit, durch eine aktivitätsgeleitete Isolierung und Identifizierung herauszufinden, welche phenolischen Inhaltsstoffe von Rotwein insbesondere für die Erhöhung der eNOS Enzym-Aktivität verantwortlich sind.

Dafür wurde der [14C]L-Arginin/[14C]L-Citrullin Konversionsassay herangezogen. Weiters konnten durch die Analysen der Phosphorylierung an der eNOS Domäne Ser^{1177} und des biologisch verfügbaren NO zusätzliche Daten gewonnen werden.

Atherosklerose stellt eine komplexe Erkrankung dar, deren Entstehung mit einer Vielzahl an Ursachen in Zusammenhang gebracht wird. Neben den klassischen Risikofaktoren für Herzkreislauferkrankungen wird von neuen, wie etwa bakterielle Infektionen, berichtet. *Chlamydia pneumoniae*, ein Gram-negatives Bakterium, ist als Verursacher von Atemwegsinfektionen bekannt. In den letzten Jahren konnte jedoch darüber hinaus in einigen Studien der Einfluss auf Atherosklerose nachgewiesen werden.

Der zweite Teil dieser Arbeit konzentriert sich auf die Isolierung und Identifizierung von neuen „anti-chlamydisch" wirkenden Inhaltsstoffen aus Rotwein, um zu untermauern, dass das Französische Paradoxon ein multikausales Phänomen ist und nur unter Berücksichtigung diverser Aspekte erklärt werden kann. Durch die Identifizierung solcher Rotwein-Inhaltsstoffe können möglicherweise neue Strategien zur Bekämpfung von Atherosklerose und Herzkreislauferkrankungen entwickelt werden.

Um sich dem Ziel der Wirkstoffidentifizierung systematisch zu nähern, wurden aus 60 repräsentativen Rotweinen acht Österreichische Rotweinproben (2x Blaufränkisch, 1x Zweigelt, 2x St. Laurent, 1x Pinot Noir, 1x Lagrein, 1x Merlot) aufgrund ihres sehr hohen bzw. sehr niedrigen Anteils an phenolischen Hauptbestandteilen ausgewählt. Dealkoholisierte Rotweinkonzentrate (DRWC) der Proben wurden zusammen mit den gewonnenen Fraktionen des Vorlaufs (FE, First Eluate) und des Rotweinphenolextrakts (RWPE) auf eNOS Aktivitätssteigerung hin untersucht.

Die Endothelzellen zeigten einen unterschiedlichen Anstieg der eNOS Aktivität nach der Behandlung mit den Zehnfach-Konzentraten der Rotweine. Hierbei konnte eine Korrelation zwischen der Aktivität und dem Gesamtphenolgehalt der acht

Rotweinproben festgestellt werden, woraus sich schließen lässt, dass der Gesamtphenolgehalt entscheidend für die gesteigerte eNOS Aktivität ist. Dennoch zeigte die Stimulierung mit einer definierten Menge an Rotweinphenolextrakt (600 µg/ml) Unterschiede im eNOS Aktivitätsmuster, was darauf hindeutet, dass das Inhaltsstoffmuster ebenfalls Einfluss auf die Aktivitätserhöhung hat.

Die mehrstufige Fraktionierung der Rotweinphenolextrakte der vier Rotweinproben mit der höchsten eNOS Aktivierung wurde durch Flüssig-Flüssig Verteilung unter Verwendung von Ethylacetat und Wasser fortgesetzt. Alle gewonnenen Apolaren Fraktionen zeigten im Gegensatz zu den meisten Polaren Fraktionen eine Erhöhung der eNOS Aktivität der Endothelzellen. Daher konzentrierte sich die weitere Fraktionierung auf die Apolare Fraktion der Rotweinprobe mit der höchsten Wirkung (Blaufränkisch 2005, Klosterneuburg).

Mittels Festphasenfraktionierung mit einer C18 Kartusche und einem Stufengradienten (Wasser/Methanol) wurden fünf Festphasenfraktionen (SPF 1 – SPF 5) hergestellt. Nur die Wirkung von SPF 3 war mit jener der Apolaren Fraktion vergleichbar. Um die Struktur eines auffallenden Inhaltsstoffes aufzuklären, musste die Fraktionierung mit einem weiteren Rotwein (Burgweingartl 2007, Klosterneuburg) wiederholt begonnen werden. Der Fraktionierungsansatz wurde modifiziert, indem anstatt der Festphasen-fraktionierung eine semi-präparative HPLC Methode (RP-18e) angewendet wurde. Schließlich führte die aktivitätsgeleitete Fraktionierung zur Isolierung des eNOS aktivierenden Inhaltsstoffes im Rotwein. Zusätzliche LC-MS/MS Ergebnisse ermöglichten die Aufklärung der Struktur dieses Inhaltsstoffes. Dabei handelt es sich um ein Gallussäurederivat konjugiert mit einem Ethylrest an der Carboxylfunktion (Gallussäureethylester), was als Ethylgallat identifiziert werden konnte.
Diese Ergebnisse weisen darauf hin, dass die Veresterung durch die Ethylgruppe von entscheidender Bedeutung für die Funktionalisierung der Gallussäure ist um die eNOS Aktivierung zu ermöglichen.

Der Einfluss von Ethylgallat auf die eNOS Aktivität in humanen Endothelzellen wird in Bezug auf Langzeitbehandlung (long term treatment) nun das erste Mal beschrieben (zwischen 10 µM und 100 µM). Darüber hinaus unterstreichen ergänzende Experimente (erhöhte Freisetzung von „bioverfügbarem“ NO und gesteigerte Phosphorylierung an Ser^{1177}) den Effekt von Ethylgallat auf das eNOS System. Diese Ergebnisse werden gestützt durch die eNOS Aktivierung von weiteren Gallussäureestern.

Weitere Experimente konnten den Einfluss des Vinifikationsprozesses insbesondere auf die Entstehung von Ethylgallat, mit Hinblick auf die eNOS Aktivierung, aufzeigen. Es konnte nachgewiesen werden, dass im nicht-fermentierten Traubensaft grundsätzlich eNOS aktivierende Inhaltsstoffe zu einem geringen Anteil vorkommen, und am Ende des Gärprozesses ein erhöhter Effekt auf das eNOS Enzym gemessen werden konnte. Demzufolge zeigte eine selektive HPLC-Analyse, dass Ethylgallat der hauptverantwortliche Bestandteil ist, der sich durch den Fermentationsprozess mit Gallussäure als Substrat im Rotwein bildet. Weitere quantitative HPLC-Analysen zeigten, dass im Gegensatz zu Rotwein (Konzentrationen von 20 µM to 70 µM) im Weißwein kein Ethylgallat vorkommt. Daraus lässt sich schließen, dass der Herstellungsprozess (bzw. die Entstehung von Ethanol) den wesentlichen Schritt für die Biosynthese von Etylgallat darstellt.

Schließlich wurde der Einfluss der Art der Rotweinlagerung auf die eNOS Aktivität untersucht, indem die Lagerung während sechs Monaten in einem Stahl- sowie einem Barriquefass verglichen wurde. Es konnte dabei kein Unterschied in der Aktivität festgestellt werden, was zeigt, dass Rotwein-Ellagitannine keine Wirkung auf die eNOS Enzymaktivität haben.

Der zweite Teil bezog sich auf eine aktivitätsgeleitete Fraktionierung von Rotwein um neue phenolische Inhibitoren von *Chlamydia pneumoniae zu finden.* Die Fraktionierung wurde analog zu dem oben erwähnten Modell mit zwei Rotweinproben

(1x Blaufränkisch, 1x Zweigelt) gestartet. Die Fraktion mit der höchsten Aktivität (AF von Blaufränkisch 2005, Klosterneuburg) wurde mittels einer semipräparativen HPLC-Methode in zehn Subfraktionen aufgetrennt

Nach der Identifizierung von mehreren Rotwein-Inhaltsstoffen wurden sowohl die Fraktionen als auch die entsprechenden reinen Inhaltsstoffe *in vitro* auf ihr Hemmpotenzial von *C. pneumoniae* hin untersucht. Während einige Fraktionen und reine Inhaltsstoffe nur moderate „anti-chlamydische" Wirkung (Wachstumshemmung und Reduktion von sogenannten Einschlüssen von *C. pneumoniae*) aufwiesen, zeigte eine Fraktion, namentlich jene, die Ethylgallat beinhaltete, eine beachtliche Wirkung gegen *C. pneumoniae*.
Es konnte eine dosisabhängige Inhibierung von *C. pneumoniae* Einschlüssen durch Ethylgallat festgestellt werden, wobei eine MIC bei 50 µM ermittelt wurde.

Zusammenfassend lässt sich sagen, dass zum ersten Mal mittels aktivitätsgeleiteter Fraktionierung die Isolierung und Identifizierung von Ethylgallat aus Rotwein gelang und seine multipotenten Eigenschaften konnten *in vitro* nachgewiesen werden. Diese Ergebnisse werden gestützt von den positiven Auswirkungen auf das eNOS System (eNOS Aktivität, Phosphorylierung von Ser^{1177}, Freisetzung von bioverfügbarem NO) und die Wirkung gegen *Chlamydia pneumoniae* (Hemmung von Wachstum und Verringerung von Einschlüssen).

7 FIGURES AND TABLES

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9 ABBREVIATIONS

AB	Aberrant body
AF	Apolar fraction
Akt	Protein kinase B
AMPK	Adenosine monophosphate-activated protein kinase
Asc	Ascorbic acid
BB 05	Blaufränkisch Burgenland 2005
BG	Butyl gallate
BH2	7,8-Dihydrobiopterin
BH4	5,6,7,8-Tetrahydrobiopterin
BK 05	Blaufränkisch Klosterneuburg 2005
BWG 07 (08)	Burgweingartl 2007 (2008)
Cai2+	Intracellular calcium concentration
CaM	Calmodulin
CaMKII	Calmodulin kinase II
Cav-1	Caveolin-1
CC	Column chromatography
CHD	Coronary heart disease
CV	Coefficient of variation
CVD	Cardiovascular disease
DAD	Diode array detector
DAF-FM	3-Amino, 4-aminomethyl-2’,7’-difluorofluorescein
DAF-FM-T	Triazolofluorescein
ddH2O	Double distilled water
DMEM	Dulbecco´s modified eagles medium
DMSO	Dimethylsuloxide
DRWC	Dealcoholized red wine concentrate
EB	Elemantary body
EC	Endothelial cells
EDRF	Endothelium-derived relaxing factor
EG	ethyl gallate
ELSD	Evaporative light scattering detector

eNOS	Endothelial nitric oxide synthase
ERK 1/2	Extracellular-signal-regulated kinases 1/2
ESI	Electrospray ionization
EtOH	Ethanol
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FE	First eluate
FMN	Flavin nucleotide
GA	Gallic acid
GATA	GATA transcription factors
GV 06	Grüner Veltliner 2006
H2O2	Hydrogen peroxide
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HL cell line	Human lung cell line
HPLC	High performance liquid chromatography
Hsp90	Heat shock protein 90
HUVEC	Human umbilical vein endothelial cells
IC50-value	Half maximal inhibitory concentration
ICAM-1	Intracellular adhesion molecule-1
IF	Immunofluorescence microscopy
IFN-γ	Interferon-gamma
IL-6 (8)	Interleukin-6 (8)
iNOS	Inducible nitric oxide synthase
IP	Isopentyl gallate
kDa	Kilodalton
LC/MS	Liquid chromatography mass spectrometry
LDL	Low density lipoprotein
LG	Lauryl gallate
L-NAME	Nω-nitro-L-arginine methyl ester
LS 04	Lagrein Südtirol 2004
MC 05	Merlot Carnuntum 2005
MeOH	Methanol

Meva	Mevastatin
MG	Methyl gallate
MIC	Minimum inhibiting concentration
MOI	Multiplicity of infection
mRNA	Messenger RNA
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappaB
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
OG	Octyl gallate
PAA	Polyacrylamide
PBS	Phosphate buffer solution
PDGF-B	Platelet-derived growth factor-B
PF	Polar fraction
PG	Propy gallate
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKG	Protein kinase G
PVDF	Polyvinylidenedifluoride
PW 04	Pinot Noir Wien 2004
RB	Reticulate body
ROS	Reactive oxygen species
RV	Reservoir volume
RWP	Red wine polyphenols
RWPE	Red wine phenol extract
SA	Syringic acid
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
SIRT1	Silent mating type information regulation 2 homologue 1
SK 05	St. Laurent Klosterneuburg 2005
SOD	Superoxide dismutase

Sp1	Pregnancy-specific beta 1-glycoprotein
Sp3	Pregnancy-specific beta 3-glycoprotein
SPE	Solid phase extraction
SPF	Solid phase fraction
SW 04	St. Laurent Wachau 2004
T/E	Trypsin/EDTA solution
TBS-T	Tris-buffered saline - Tween 20
TF	Tumor factor
TGF-β1	Tumor growth factor-β1
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor-α
TR-FIA	Time-resolved fluorometric immune-assay
VCAM	Vascular adhesion molecule-1
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
WWPE	White wine phenol extract
ZC 05	Zweigelt Carnuntum 2005

10 CURRICULUM VITAE

Personal details

Name:	Mag. pharm. Oliver Donath
Date and place of birth:	August 13th 1978, Ried im Innkreis
Nationality:	Austria
Email:	oliverdonath@gmx.at

Employment

01/2012 – 11/2014	Analytical chemist in the R&D laboratory for veterinary medicinal products at Richter Pharma AG, 4600 Wels, Austria
09/2006 - present:	PhD student at the Department of Pharmacognosy, University of Vienna ➢ Influence of polyphenols from Austrian red wines on the endothelial nitric oxide synthase system
11/2005 – 12/2011:	Employment as technical assistant at the Department of Pharmacognosy, University of Vienna
WS2007/08 - WS2010/11:	*Co*-supervision of three MSc. theses
SS2009 – SS2011:	Assistant in the practical course "Pharmaceutical quality of biogenic drugs"
SS2006 - WS2008/09:	Tutor in the practical course "Pharmaceutical quality of biogenic drugs" and "Extraction and phytochemical analysis of drugs"
WS2005/06:	Tutor in the practical course "Introduction to microbiology"
03/2005 - 06/2005:	Diploma thesis at the Department of Pharmacognosy, University of Vienna ➢ Phytochemical investigation of *Myricaria longifolia* – a plant used in traditional Mongolian medicine

Research experience

(Phyto)chemistry:	Extraction, fractionation, isolation, characterization and structure elucidation of small molecules out of diverse matrices using conventional and state-of-the-art equipment (SPE, TLC, Column chromatography, HPLC, UHPLC, LC-MS/MS, GC-MS, IR, NMR). Quantification and stability investigations of

	natural compounds and various pharmaceuticals (e.g. antibiotics, antimycotics) via HPLC and LC-MS/MS Formulation studies (e.g. injectables containing analgetics), development of analytical methods and validation (especially HPLC); compilation of validation reports, SOPs and quality control plans, stability testing of various products in a GMP regulated laboratory
Cell culture:	Cultivation and handling of cell lines and primary cells (EA.hy926; HUVEC, HAEC), western blotting, eNOS activity and NO-release measurement ([^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay, DAF-FM assay), relevant statistics

Acquired Grants

03/2008 – 03/2011:	HJST-Project H2317/2007: "Impact of polyphenolic compounds from Austrian red wines on health – influence on eNOS and antioxidant capacity"

Additional skills and competences

10/2013	Seminar "HPLC method development in a GMP laboratory"
07/2013	Workshop "Teambuilding and management skills"
06/2013	"GMP in the Laboratory" (Training)
04/2012	Workshop "Management skills"
05/2011:	Training course on "UHPLC/MS/MS application of clinical samples"
02/2011:	22nd MassSpec-Forum-Vienna-2011 (2 days), Faculty of Chemistry, Vienna
02/2011:	28th Agilent Forum Analytik (2 days), Schloß Wilhelminenberg, Vienna
11/2010:	„Dionex HPLC Analytic Club", Vienna
09/2010:	ÖPhG/ASAC – summerschool (4 days), Schloß Seggau, "Advances in hyphenated LC/MS techniques and methods"
04/2009:	Organising committee member of the "21st Meeting of the Austrian Pharmaceutical Society (ÖPhG)", Vienna
02/2009:	3-day training course on "Ion Trap – low molecular weight applications"
02/2009:	3-day training course on "API 4000^{TM} – LC/MS/MS applications"
09/2008:	ÖPhG/ASAC – summerschool (4 days), Schloß Seggau, "Chromatography and mass spectrometry"
Computer skills:	Windows, MS Office (Microsoft Word, Powerpoint, Excel), HPLC software (Chromeleon, Lab Solution), MS (Analyst software), ChemOffice (ChemDraw Ultra, Chem3D Pro), Scifinder, Beilstein crossfire, Nmrpredict, Nmrdb, GraphPad software, Endnote

Education

09/2006 - present:	PhD student at the Department of Pharmacognosy, University of Vienna
10/1998 – 08/2006:	Studies of Pharmacy, University of Vienna
10/1997 – 05/1998:	Military service; Ried im Innkreis, Austria
09/1997:	European Baccalaureate; Ried im Innkreis, Austria

Languages

German:	Mother tongue
English:	Fluent in writing and speaking
French:	High school level

Interests

Hobbies:	mountainbiking, mountaineering, snowboarding, climbing, sailing, cooking, ultimate frisbee, squash, music

11 RESEARCH

Projects

Responsible researcher in HJST-Project H2317/2007: „Impact of polyphenolic compounds from Austrian red wines on health – influence on eNOS and antioxidant capacity“

Recent publications

Donath O, Zehl M, Ladurner A, Waldbauer K, Schmitt CA, Wendelin S, Eder R, Reznicek G, Dirsch VM. Activity Profiling of Austrian Red Wine Targeting Cardiovascular Diseases: a Contribution to Understand the “French Paradox”. (In preparation)

Salin O, Donath O, Ladurner A, Näreoja T, Dirsch VM, Reznicek G, Vuorela P. Red Wine Inhibits *Chlamydia pneumoniae* Growth and Normalizes eNOS Levels in vitro – a Dual Connection to Atherosclerosis. (In preparation)

Poeppl W, Lingscheid T, Bernitzky D, Schwarze UY, Donath O, Perkmann T, Kozakowski N, Plasenzotti R, Reznicek G, Burgmann H. Efficacy of Fosfomycin Compared to Vancomycin in Treatment of Implant-Associated Chronic Methicillin-Resistant Staphylococcus aureus Osteomyelitis in Rats. Antimicrobial Agents and Chemotherapy, Volume: 58 Issue: 9 Pages: 5111-5116; 2014.

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Koller VJ, Dirsch VM, Beres H, Donath O, Reznicek G, Lubitz W, Kudela P. Modulation of bacterial ghosts - induced nitric oxide production in macrophages by bacterial ghost-delivered resveratrol. FEBS Journal, Voume: 280 Issue: 5 Pages: 1214-1225; 2013.

Kainz K, Virtbauer J, Kählig H, Arion V, Donath O, Reznicek G, Huber W, Marian B, Krenn L. Two Unusual Methylenecylcopropane Glucosides from Metaxya rostrata C.Presl. Helvetica Chimica Acta, Volume: 95 Issue: 9 Pages: 1531-1537; 2012.

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Poeppl W, Zeitlinger M, Donath O, Wurm G, Müller M, Botha F, Illievich U, Burgmann H. Penetration of doripenem in human brain: an observational microdialysis study in patients with acute brain injury. International Journal of Antimicrobial Agents, Volume: 39 Issue: 4 Pages: 343-345; 2012.

Vogler G, Donath O, Saukel J, Rauch A, Kählig H, Krenn L. Polar phenolic compounds in Dryopteris filix-mas and Dryopteris dilatata. Verhandlungen der Zoologisch-Botanischen Gesellschaft in Österreich, Volume: 148 Pages: 279-289; 2012.

Burian B, Zeitlinger M, Donath O, Reznicek G, Sauermann R. Penetration of doripenem into skeletal muscle and subcutaneous adipose tissue in healthy volunteers. Antimicrobial Agents and Chemotherapy, Volume: 56 Issue: 1 Pages: 532-535; 2012.

Virtbauer J, Krenn L, Kählig H, Hüfner A, Donath O, Marian B. Chemical and Pharmacological Investigations of Metaxya rostrata. Z. Naturforsch. C, 63:469-75, 2008.

Donath O, Schmitt CA, Wendelin S, Eder R, Reznicek G, Dirsch VM. Influence of fractions from Austrian red wines on the endothelial nitric oxide synthase. Polyphenols Communications 2008, 699-700, 2008.

Waldmann A, Donath O, Eder R, Krenn L. Antioxidant Properties of Austrian Red Wines. Polyphenols Communications 2008, 559-560, 2008.

Short lectures

Donath O, Reznicek G. Speed-Up of HPLC-methods from the European Pharmacopoeia. Workshop Dionex Co., Vienna (Austria), 2009. (Invitation).

Donath O, Eder R, Reznicek G, Dirsch VM. Austrian red wine samples reveal enhanced activity of the enzyme endothelial nitric oxide synthase. 21st Meeting of the Austrian Pharmaceutical Society (ÖPhG), Vienna (Austria), 2009. (Invitation)

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Poster contributions

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