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Experimental study on the influence of phenolic compound structures on antioxidant activity measurement methods

Melanie Platzer, M.Sc.

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Abstract

Oxidation sensitive products based on vegetable oils are often protected by antioxidants. Within the scope of this dissertation, results of different measurement methods for determining the antioxidant effect of polyphenols were compared and their structural influences on the measurement results were investigated. Thus, it was possible to identify highly effective plant antioxidant additives and to replace time-consuming, application-oriented oil measurement methods by faster *in vitro* assays.

Kurzfassung

Oxidationsempfindliche Produkte auf Basis pflanzlicher Öle werden häufig durch Antioxidantien geschützt. Im Rahmen dieser Dissertation wurden Ergebnisse verschiedener Messmethoden zur Bestimmung der antioxidativen Wirkung von Polyphenolen verglichen und deren strukturelle Einflüsse auf die Messergebnisse untersucht. So war es möglich hochwirksame pflanzliche Oxidationsschutz-Additive zu identifizieren und zeitaufwendige, anwendungsnahe Ölmessmethoden durch schnellere *in vitro* Assays zu ersetzen.

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Abbreviations

Abbreviations

AAPH	2,2’-azobis-(2-methylpropionamidine)-dihydrochloride
ABTS	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
BHA	butylhydroxyanisol
BHT	butylhydroxytoluol
CUPRAC	cupric ion reducing antioxidant capacity
DMPD	N,N-dimethyl-p-phenylenediamine
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	differential scanning calorimetry
$\mathbf{EC}_{50}$	half maximal effective concentration
FA	fatty acid
FC	Folin-Ciocalteu
FRAP	ferric reducing antioxidant power
GC	gas chromatographie
HAT	hydrogen atom transfer
HPLC	high performance liquid chromatography
HP-SEC	high perfomance size exclusion chromatography
ORAC	oxygen radical absorbance capacity
PC	principle component
P-DSC	pressurized differential scanning calorimetry
PG	propyl gallate
QSPR	quantitative structure-property relationship
SET	single electron transfer
SET-PT	single electron transfer followed by a proton transfer
SPLET	sequential proton loss electron transfer
TBHQ	*tert*-buthylhydroquinine
TEAC	trolox equivalent antioxidant capacity
$\mathbf{T}_{EC50}$	time to reach EC_{50}
TRAP	total radical trapping antioxidant parameter

Scientific contributions

Peer reviewed publications with first authorship, which are part of this thesis

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2. Platzer M., Kiese S., Herfellner T., Schweiggert-Weisz U., Eisner P., How does the phenol structure influence the result of the Folin-Ciocalteu Assay?, *Antioxidants* **10**, 811 (2021)

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1 Introduction

Lubricants facilitate movement between surfaces in contact with each other in order to reduce friction, wear and heat, thus extending the operational lifetime of machinery [1]. Since the beginning of the industrial revolution, the demand for lubricants increased significantly, and in order to save costs, lubricants were made from mineral oil since the mid-19th century [2, 3]. Today, 95 % of all lubricants are based on mineral oils, which are a mixture of hydrocarbons and kerosene, naphthenes and aromatics [4, 5].

However, fossil resources are limited, which is becoming problematic since the demand is continuously rising. In addition, the use of mineral oils from different sources leads to variations in the composition and differences from batch to batch. The biggest issue with their use, however, is certainly that these substances are not biodegradable and pose a risk to the environment, which is additionally associated with costly and time-consuming disposal [4–7]. Furthermore, due to their high toxicity, mineral oils are classified as hazardous to health and in some cases even as carcinogenic [4]. Since lubricants can be used in both open and closed systems, about 50 % of the lubricants used are released into the environment, which is equivalent to 200,000 t/a. In addition, low molecular weight volatiles can be released into the atmosphere causing additional negative environmental impact [3, 4, 6, 7].

As a result of the many disadvantages and due to factors like climate change and environmental awareness, the interest in alternative base media continues to grow [1]. Commercially available biolubricants usually consist of a mixture of vegetable oils and synthetic esters, which are partly derived from fossil oil. Thus, a biolubricant is defined only by its low toxicity and biodegradability and not by its origin. Examples are polyalklene glycols, low-viscosity polyalphaolefins, acid esters, polyol esters and high-oleic or highly unsaturated vegetable oils. Although vegetable oils are more expensive, they offer lots of advantages and therefore may provide an alternative to conventional base media. These include biodegradability, high lubricity, flash point and viscosity index, as well as low friction, wear and toxicity [3, 4, 8]. Which kind of vegetable oil is used for the production of bio-based lubricants depends mainly on geographical factors, but speaking of the

European market, sunflower and rapeseed oils are primarily used [3, 9]. Due to its composition and advantageous tribological properties, rapeseed oil is particularly popular for the use as a lubricant and its advantageous tribological properties [3, 10]. However, vegetable oils also show disadvantages, such as poor cold flow properties, susceptibility to hydrolysis caused by the presence of water, as well as low temperature stability, resulting in deteriorated tribological behavior and increased viscosity [3, 11]. Above all, they have very low oxidation stability, which makes them unsuitable for the use as lubricants in their native form [12–14]. Due to the aforementioned negative aspects, many researchers are working on the improvement of vegetable oils to make them suitable as base media. This is done, on the one hand, by adding various additives and, on the other hand, by optimizing the processing conditions to improve the thermal-oxidative stability of vegetable oils [14, 15].

The use of additives, like wear protection, corrosion protection, performance and oxidation protection additives as well as friction reducers, helps to meet the requirements of high-performance lubricants. To date, these additives are mostly synthetic fossil-based substances, which are used in concentrations of up to 10 %. As mentioned above, especially in the area of media based on vegetable oils, the oxidation protection additives are of enormous importance [14, 16]. However, conventional antioxidant additives, like propyl gallate (PG), *tert*-buthylhydroquinine (TBHQ), butylhydroxytoluol (BHT) or butylhydroxyanisol (BHA), are classified as hazardous to health and carcinogenic, which is why the trend in recent years is moving towards natural antioxidants [17–22].

In this context, plant based ingredients can be a valuable source of natural antioxidants. Most of them are phenolic compounds, ranging from low molecular weight substances such as phenolic acids to the more complex flavonoids, which are usually obtained by a simple solvent-based extraction process [23–29]. The extracts are rich in secondary metabolites, which protect plants from environmental factors such as predators, pests, pathogens and UV radiation [30–32]. These substances are already used in numerous products, for example in the food and dietary supplement industry, where they are considered to have a positive effect on health, prevent cancer and metabolic syndromes and reduce the risk of cardiovascular diseases [17–22, 30, 33–37]. It has also been shown that antioxidants obtained from side streams of the agricultural and food industry, such as seeds or shells, partly show a higher antioxidant activity than synthetic antioxidants [22, 38]. In addition to their health-promoting properties, they are able to scavenge free radicals and thus protect fatty foods and oils from oxidation [30, 33–37].

For the determination of the antioxidant activity and the polyphenolic content, various *in vitro* assays have been developed. Among the most commonly used methods are 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu (FC), and the oxygen radical absorbance capacity (ORAC) assay [39–42]. Although there have been carried out many studies based on these assays and they are frequently used to determine the antioxidant activity of plant extracts, it is not clear which of the antioxidant assays is best suited for which type of phenolic substance, and thus for which kind of extracts. Furthermore, different measurement and evaluation methods exist, which makes it difficult to compare studies from the literature about the structure-activity relationships between the phenolic substances and the used model radicals of the assays. Also, a comparison between the results of the *in vitro* assays with test methods suitable for application media such as oils, has not been adequately described in the literature. To determine the effects of antioxidants in oil, especially in technical areas such as the lubricant industry, time-consuming and cost-intensive application-based methods such as rancimat method and pressurized differential scanning calorimetry (P-DSC) measurements are performed. Therefore, there is an increasing need for low-cost, quick and easy-to-perform methods which, moreover, do not require expensive laboratory equipment [43]. To meet this demand, this thesis focuses on the correlation of the results from *in vitro* assays with the antioxidant effect in rapeseed oil.

2 State of the Art

The following chapter describes the state of knowledge this thesis is based on, the classification and production of vegetable oils and fats as well as processes of fat oxidation. Special attention is paid to the protection against oxidation by the addition of antioxidants. Furthermore, methods of measuring oxidation stability are described. With regard to the use as bio-based lubricants, conventional additives and their mechanisms of action are considered, as well as bio-based alternatives from plant ingredients. Furthermore, different methods of the determination of the antioxidative capacity of natural phenolic compounds as well as their advantages and specific limitations are indicated.

2.1 Vegetable fats and oils

Vegetable fats and oils are usually obtained from seeds (e.g. rapeseed, soybeans and sunflower) or pulp of fat-rich plants (e.g. palm, olive, macauba and avocado) [44–46]. The most produced vegetable oils in the world are palm (76.5 mio. t/a), soybean (61.9 mio. t/a), rapeseed (28.1 mio. t/a), sunflower (22.1 mio. t/a), palm kernel (8.8 mio. t/a), peanut (6.5 mio. t/a), cottonseed (5.1 mio. t/a), coconut (3.6 mio. t/a), and olive oil (3.3 mio. t/a) [47].

Natural fats and oils consist mainly of triglycerides, which are esters of the trivalent alcohol glycerol and fatty acid (FA). Oils are composed of different FA (Table 2.1), which can be influenced by factors such as cultivation conditions and climate [44]. Most of the FA are predominantly unbranched and have an even number of carbon atoms. In most cases, the number of carbon atoms ranges from 4 to 26. Vegetable fats are usually esterified with a saturated fatty acid at C_1 and C_3 of the glycerol and an unsaturated fatty acid at C_2. However, there are also exceptions, such as coconut fat, whose structure is more similar to animal fat and consists mainly of saturated FA [49–51]. The chain length and the number of double bonds of the FA as well as the position on the glycerol molecule determine the properties of the triglycerides as well as the polarity and the melting point [48]. Based on the properties at room temperature and thus their aggregate state, a distinction is made between fats and oils. While a liquid state is defined as oils,

Table 2.1: Overview of naturally occurring FA from different vegetable fats and oils (translated and reproduced from Matissek and Baltes [48] with some modifications).

common name	**name**	**chemical structure**	**occurence**
saturated FA			
caproic acid	hexanoic acid	$C_5H_{11}COOH$	palm kernel fat, coconut fat
caprylic acid	octanoic acid	$C_7H_{15}COOH$	coconut fat, palm kernel fat
capric acid	decanoic acid	$C_9H_{19}COOH$	coconut fat, palm kernel fat
lauric acid	dodecanoic acid	$C11H_{23}COOH$	coconut fat, palm kernel fat
myristic acid	tetradecanoic acid	$C_{13}H_{27}COOH$	almost all vegetable fats
palminic acid	hexadecanoic acid	$C_{15}H_{31}COOH$	all vegetable fats
stearic acid	octadecanoic acid	$C_{17}H_{35}COOH$	cocoa butter
arachidic acid	eicosanoic acid	$C_{19}H_{39}COOH$	peanut fat
behenic acid	docosanoic acid	$C_{21}H_{43}COOH$	peanut fat, canola oil
mono unsaturated FA			
palimtiolic acid	9-hexadecenoic acid	$C_{15}H_{29}COOH$	little in vegetable fats
oleic acid	9-octadecenoic acid	$C_{17}H_{33}COOH$	all vegetable fats
erucic acid	13-docosenoic acid	$C_{21}H_{14}COOH$	cruciferous fats
poly unsaturated FA			
linoleic acid	9,12-octadecadienoic acid	$C_{17}H_{31}COOH$	safflower, soybean, sunflower and cottonseed oils
linolenic acid	9,12,15-octadecatrienoic acid	$C_{17}H_{29}COOH$	linseed oil

which consist mainly of unsaturated FA, a solid state is referred to as fats, wich are mainly composed of saturated FA. [44, 52–55]. Additionally to triglycerides, vegetable oils also contain monoacylglyceroles and diacylglyceroles, phosphatides, tocopherols, fatty alcohols, free FA, sterols, pigments and tocotrioles [56].

In the production of vegetable fats and oils, mainly cold pressing and solvent extraction processes are used. In cold pressing, after conditioning by adjusting the temperature to 40 °C to 60 °C and relative humidity, the seeds and fruits are ground and then mechanically pressed in a multi-stage process [51, 57–59]. During extraction process, the fruits and seeds are also first ground and then extracted using solvents, such as hexane, which is subsequently seperated from the oil by desolventation [51, 59]. After the separation of solvents, a refining process takes place where undesirable odors and flavors are removed and the fats and oils are deacidified, deodorized, decolorized and steamed. For the deacidification alkynes or glycerol are added in order to neutralize or esterify the free FA. Subsequently, the fats and oils are treated with solid absorbers such as activated carbon for decolorization. In the final refining step, unwanted odors and flavors are separated by steam distillation under vacuum [51, 59]. An advantage of the extraction process compared to the pressing process is a higher production yield. However, this process also has disadvantages, such as the removal of the solvent. Afterwards, the oils are refined with negative effect on the quality of the oil, i.e. loss of valuable ingredients such as minerals, vitamins, and carotenoids. However, refining also brings advantages, such as long shelf life, high heat and long-term stability [50, 59, 60]. In modern refining processes, it should already be possible to purify the oils without loss of natural flavor, so that they do not differ in taste from cold pressed oils [51, 59]. Therefore, cold pressed, unrefined oils are considered to be of higher quality and are used especially in the food, cosmetic and pharmaceutical industry [50, 51, 60].

Since the enviromental awareness of consumers has increased significantly in recent years, vegetable oils are also used in technical areas, for example in the lubricant industry to replace mineral oils. Fully refined vegetable oils are used for this purpose [4], which is why refined rapeseed oil was used for all tests within this thesis.

2.1.1 Shelf life and oxidation of lipid systems

The degradation of fats and oils can take place in different ways, distinguishing between enzymatically catalyzed oxidation, chemically catalyzed autoxidation and non-enzymatic, non-radical photooxidation [17, 61]. For the shelf life of vegetable oils the chemically catalyzed autoxidation is important, which is influenced by light, oxygen partial pressure, heavy metals, water content of the oil, and temperature [61]. In the following, this process is explained and the reaction steps are shown.

Autoxidation

During autoxidation, a sequence of chemical reactions takes place, which is divided into three main steps: initiation, chain propagation and chain termination [17, 48, 52].

During the **chain initiation**, the formation of an alkyl radical (L·) occurs by cleavage of a hydrogen atom from the unsaturated lipid (LH) due to the presence of an initiator (I·) such as singlet oxygen [48],

$$\mathrm{LH} \xrightarrow{\mathrm{I}\cdot} \mathrm{L}\cdot + \mathrm{IH}. \tag{2.1}$$

These reactions can also be catalyzed by the presence of heavy metal ions such as copper, nickel or iron [48]. The release of a hydrogen atom depends on the bond dissociation energy, which is low at positions next to alkene groups [17, 62]. The radicals formed are short lived and unstable substances which stabilize by binding a hydrogen atom. This step of the oxidation proceeds slowly, due to the low concentration of radicals, and accelerates at the end of the initiation step, which is accompanied by an increased oxygen consumption and peroxide production [63].

In the **chain propagation** step, the previously formed unstable alkyl radicals react with oxygen to form peroxide radicals (LOO·) (Reaction 2.2),

$$\mathrm{L\cdot + O_2 \longrightarrow LOO\cdot.} \tag{2.2}$$

This reaction has a low activation energy and therefore proceeds very rapidly [17, 62–64].

The peroxide radicals formed are highly reactive substances, which attack other unsaturated lipids and thus form hydroperoxides (LOOH) by the uptake of hydrogen atoms [17, 52, 62, 63, 65],

$$\mathrm{LOO\cdot + LH \longrightarrow LOOH + L\cdot.} \tag{2.3}$$

This reaction is also called a "self-sustaining" radical chain reaction, because in this irreversible step, a large amount of hydroperoxides is formed very quickly. Stabilization of the radicals is usually accomplished by the delocalization of electrons [63, 65].

Furthermore, the radicals can also decompose into secondary, nonradical compounds, resulting in the formation of volatile hydrocarbons, aldehydes, alcohols and ketones. Non-volatile substances can also be formed, which include polymers, triacylglycerides and non-volatile aldehydes. [17, 52, 63].

The final step of autoxidation is the **chain termination**, where two radicals are combined to form a stable, non-radical product, such as alkyl or peroxyl radicals or a mixture of both [17, 52].

The chain termination can occur by condensation of alkyl, peroxyl or alkoxyl radicals. Furthermore, radicals can combine at low temperatures to form peroxyl-linked dimers (LOOL) [17, 66],

$$\mathrm{2\,LOO\cdot \longrightarrow LOOL + O_2,} \tag{2.4}$$

$$\mathrm{LOO\cdot + L\cdot \longrightarrow stable\,product.} \tag{2.5}$$

At elevated temperatures and low oxygen pressure, alkyl and alkoxyl radicals can form ether-containing dimers (Reaction 2.6) or carbon-carbon linked dimers

(Reaction 2.7) [17, 66],

$$\mathrm{LO\cdot + L\cdot \longrightarrow LOL,} \tag{2.6}$$

$$\mathrm{2\,L\cdot \longrightarrow L{-}L.} \tag{2.7}$$

Inhibition of autoxidation in lipid systems

A common method to interrupt the autoxidation chain reactions is the use of antioxidants [17], which can react in two different mechanisms and are therefore divided into secondary and primary antioxidants.

Primary antioxidants, also known as "chain-breaking" or "type-1" antioxidants, prevent oxidation by releasing a hydrogen atom or electron, thus interrupting initiation or chain propagation. The binding of the hydrogen atom leads to the neutralization of a peroxyl (Reaction 2.8), alkoxyl (Reaction 2.9), or alkyl (Reaction 2.10) radical, turning the antioxidants into radicals A· themselves [17, 67–69],

$$\mathrm{LOO\cdot + AH \longrightarrow LOOH + A\cdot,} \tag{2.8}$$

$$\mathrm{LO\cdot + AH \longrightarrow LOH + A\cdot,} \tag{2.9}$$

$$\mathrm{L\cdot + AH \longrightarrow LH + A\cdot.} \tag{2.10}$$

Reaction 2.10 occurs mainly at increased temperature and low oxygen partial pressure, whereas at atmospheric pressure the alkyl radical is converted into a peroxyl radical due to its short lifetime [17, 67–69].

The antioxidant radical A· reacts either slowly with an unsaturated lipid (Reaction 2.1) or rapidly with another peroxyl radical (Reaction 2.11), an alkyl radical (Reaction 2.12), or stabilizes itself by forming a nonradical compound A-A by electron delocalization (Reaction 2.13) [17, 67–69],

$$\mathrm{A\cdot + LOO\cdot \longrightarrow LOOA,} \tag{2.11}$$

$$\mathrm{LO\cdot + A\cdot \longrightarrow LOA,} \tag{2.12}$$

$$\mathrm{A\cdot + A\cdot \longrightarrow A{-}A.} \tag{2.13}$$

Primary antioxidants have a low affinity to lipids and a high affinity to peroxyl radicals, which is why the rate of propagation is lower. In most cases, they are substances with mono- or polyhydroxy-phenol groups and their activity depends on the resonance stabilization of the phenoxyl radicals. Accordingly, an electron donating side group in *ortho* or *para* position increases the activity, due to resonance stabilization of the resulting quinone structure [17, 67, 68]. Since the primary antioxidants affect chain initiation, they should be added before the autoxidation process starts, otherwise they will be rapidly consumed by the developed peroxides [67].

Secondary antioxidants, also called ”preventive” or ”type-2” antioxidants, slow down the oxidation rate. Unlike primary antioxidants, they do not scavenge radicals, but prevent prooxidant factors, by absorbing UV radiation or deactivating singlet oxygen. In addition, they can form chelates together with metals, whereby these have no longer a catalytic effect on the oxidation process. Although the secondary antioxidants have no influence on the chain reaction, they can positively affect the primary antioxidants by regenerating them and, in some cases, enhancing their antioxidant activity [67, 70].

The protection of oils against oxidation plays a decisive role not only in the food industry but also in technical areas such as the chemical industry. If the base oils used in lubricants do not possess sufficient oxidation stability, the shelf life can be improved by the addition of oxidation protection additives, which will be discussed in more detail in the following section.

2.1.2 Oxidation protection additives for vegetable fats and oils in the lubricant industry

Antioxidant additives are divided into primary and secondary antioxidants in the lubricant industry and are usually used as mixtures [3, 71–74]. These include both natural (tocopherol, acorbyl palmitate, propyl gallate) and synthetic antioxidants (BHT, BHA, TBHQ, 4,4’-methylenebis(2,6-di-tert-butylphenol)), which are also present in other bio-based lubricants [3, 73–75]. Some of the commercially available lubricant additives contain pollutants such as heavy metals, chlorine and phosphorus, and should be substituted. This leads to an increasing demand for environmentally friendly additives [3, 76, 77]. However, so far the use of bio-based additives is only part of research and they are not used in industry. One of these studies by Kumar et al. (2020) deals with the use of different natural extracts rich in antioxidants such as ginger, garlic and pepper and achieved first positive results [78]. Responsible for the antioxidant effect in these plant extracts are the contained secondary plant metabolites, which are of crucial importance for this work and are therefore presented in Chapter 2.3. Prior to this, the various measurement methods of determining oxidation stability of fats and oils are discussed in more detail.

2.2 Oxidation stability determination of lipid systems

There are different methods to determine the oil stability and the most common ones are [17, 79–89]:

- sensory analysis
- spectrophotometric analyses
- chromatographical methods: gas chromatographie (GC), high performance liquid chromatography (HPLC), high perfomance size exclusion chromatography (HP-SEC)
- fluorescence measurements
- octanol method
- conjugated chromophores
- iodine value
- Schaal oven test
- active oxygen method
- pressurized differential scanning calorimetry (P-DSC)
- rancimat method

In this thesis, the measurements using P-DSC and rancimat method were used, which is why these methods are described in more detail below.

Rancimat method

The rancimat method is one of the most widely-used methods of measuring the oxidation stability of oils [80, 90]. In addition to the food industry, it is also commonly used for biodiesel analysis, as this method meets the two european standards of the biodiesel industry (EN 14213 for residential heating oil and EN 14214 for automotive diesel fuel) [91].

One reason why rancimat device is so widespread is the fact that it is an automated apparatus. A schematic representation of the rancimat apparatus is shown in Figure 2.1 [80, 90].

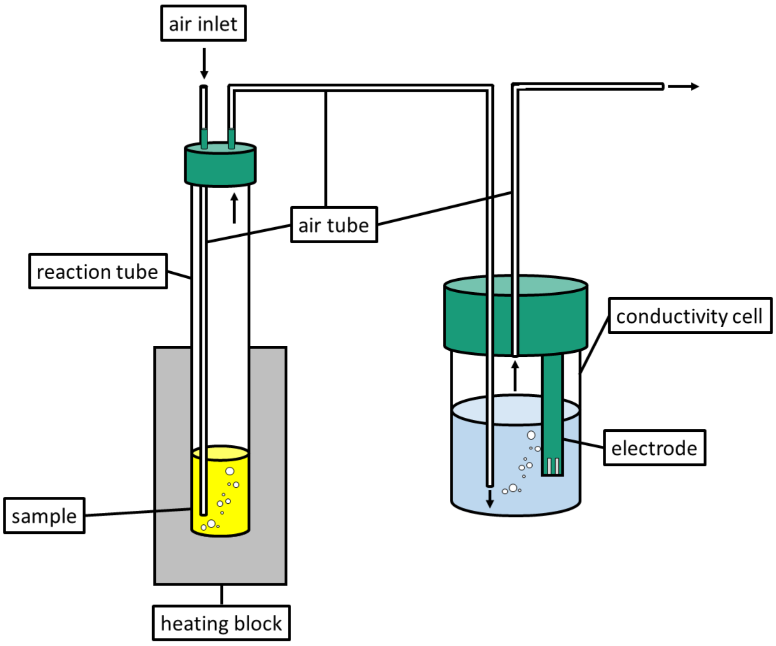

Figure 2.1: Schematic representation of a rancimat measuring cell reproduced from [82, 91–93].

The rancimat method can be performed at different conditions and thus can be adapted to samples from different applications. To do so, the samples are placed in a reaction tube which is heated to a constant temperature between 50 and 220 °C [94]. An air flow of 10 to 20 $\mathrm{L\,h^{-1}}$ is directed into the heated samples and transfers the volatile degradation products into a conductivity cell, which is filled with demineralized water. There, the conductivity is detected during the entire measurement by an electrode. The end of the measurement is reached as soon as the conductivity starts to increase rapidly which can be attributed to the formation of the secondary oxidation products, i.e., the volatile carboxylic acids. As a result, the conductivity as a function of time is obtained and the oxidation induction time is calculated either by deriving the curve two times or by using the tangent method [80, 82, 90, 95, 96]. Here, two tangents are created manually and their intersection point is determined. The first tangent is drawn along the flat part of the curve and the second tangent along the fast rising part. An example evaluation is shown in Figure 2.2 [97].

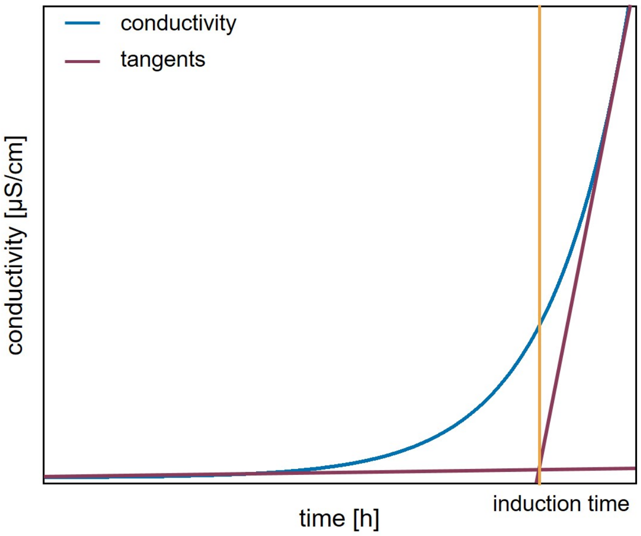

Figure 2.2: Schematic representation of the determination of oxidation induction time using the tangent method.

Pressurized differential scanning calorimetry (P-DSC)

Thermal analysis can be used to monitor the oxidative stability of animal and vegetable oils and fats [82, 96, 98–102]. These include, for example, differential scanning calorimetry (DSC), which has a wide range of applications and is used, among others, for the determination of oxidation induction time in the biodiesel and lubricant industry [103–105]. The method is based on the measurement of various properties such as specific heat, thermo-oxidative behavior, activation energy of thermal decomposition, temperature, and enthalpy of crystallization, degree of unsaturation using melting and crystallization oil profile curves and the effect of antioxidants on the thermal stability of oils [99].

During measurement, an increased temperature leads to thermal transition of a FA. The resulting reaction of an unsaturated FA with an oxygen molecule is an exothermic reaction that leads to a decrease in enthalpy, from which the oxidation stability of vegetable oils can be determined [103, 106]. The oxidation induction time then indicates the time interval from exposure to oxygen until exothermic oxidation, at constant temperature and pressure, as shown in Figure 2.3 [107–109].

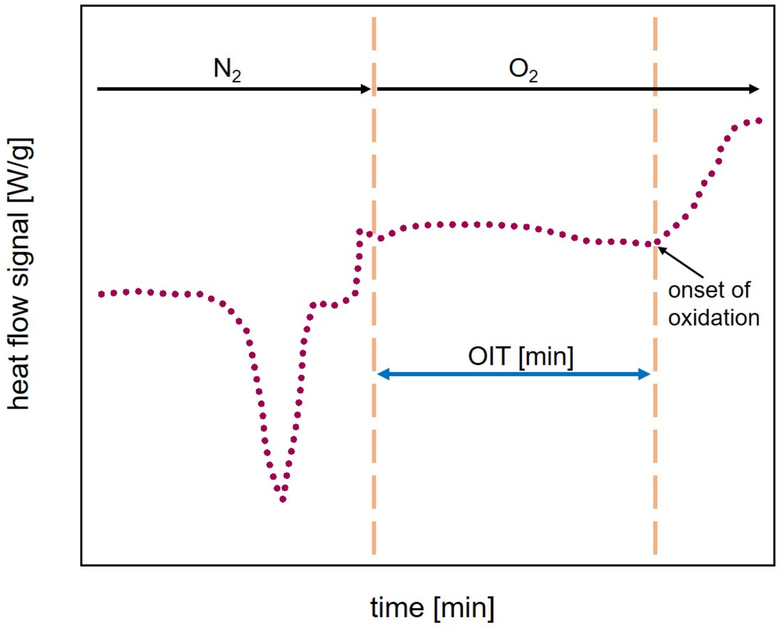

Figure 2.3: Exemplary representation of a measurement curve for determining oxidation induction time using P-DSC measurement. Reproduced with some modifications [66, 110].

A newer variant of DSC, P-DSC, is also used to measure the oxidation induction time. Increasing the pressure during the measurement has several advantages, such as shortening the measurement times and suppressing pressure-sensitive reactions (volatilization of substances) [105, 111–117].

2.3 Natural antioxidants

Plants produce a wide range of metabolites throughout their life cycle, which can be divided into primary and secondary plant metabolites. Primary plant metabolites play an important role in photosynthesis, growth and development of the plant and include, for example, common sugars, amino acids and chlorophyll.

However, the majority of the plant metabolites are produced in the secondary metabolic pathway and are not crucial for plant growth and development, but play a key role in protecting the plant from microbial infections, viruses and UV radiation, or serve as signaling substances to attract seed dispersing or pollinating animals. The content of secondary plant substances is partly genetically determined, but also partly controlled by environmental adaptations caused by biotic and abiotic stress [31, 32, 118–125]. Due to an adaption of plants to changing environmental conditions in the course of the evolution, a multiplicity of different secondary plant materials developed. The number of different substances is estimated at over 100,000, from which only a fraction is known nowadays [119, 122, 125–129]. Due to their different structural and chemical properties, secondary plant compounds can be used in various fields, including for example, the use in pharmaceutical products, but also the use in novel foods or in technical applications [130].

Secondary plant compounds are divided into different classes based on their composition, chemical structure or their solubility in different solvents, as well as their way of synthesis. Often they are devided into the main classes: steroids, terpenes and polyphenols, based on their structural an chemical properties [131–133]. Since only the class of polyphenols is important for this work, it will be considered in more detail below.

2.3.1 Polyphenols

Polyphenols are very common in the plant kingdom and are among the most important secondary plant compounds. They are found in various plants, both edible and non-edible [24, 126, 134]. Polyphenols are formed either in the polyketide-acetate/malonate pathway or the shikimate/phenylpropanoid pathway [24, 125, 135]. They occur in fruits and vegetables, where they are among others, responsible for the color and flavor [134]. Most of them are water-soluble and due to their health-promoting properties, polyphenols are recommended in human nutrition [24, 125, 136]. An intake occurs through the consumption of fruits and vegetables, but also through beverages such as coffee and tea. In fruits or also in fruit juices they are mainly responsible for the partly bitter and astringent taste [24].

Polyphenols are usually isolated from plant materials by solid-liquid extraction using different solvents that can penetrate the cell walls and thus release the polyphenols [137]. To extract both the free and bound polyphenols, two-step extractions can be performed, which include acid treatment in addition to solvent extraction [138]. The effectiveness of the extraction process depends on the interaction of the solvent with the cell wall and the diffusion time. However, the extraction parameters also have an influence on the extraction yield. Moreover, since the optimum conditions differ from raw material to raw material, they have to be adjusted before each extraction. The most influential parameters include the particle size of the raw material, type of solvent, extraction time, temperature, and the ratio of the solvent to the sample as well as to the water contained [139]. In addition to conventional solid-liquid extraction, there are numerous alternatives for the recovery of polyphenols, including ultrasonic- and microwave-assisted extraction, extraction with supercritical fluids, and liquid extraction under pressure [140].

Previously, polyphenols were defined as substances consisting of more than one aromatic ring, with one or more hydroxyl substituents per ring [141]. However, this definition is very imprecise, because it would also include substances such as the hormone estrone. For this reason, the definition of polyphenols has continued to expand over the years. Meanwhile, it also includes smaller molecules such as phenolic acids, consisting of only one aromatic ring. Besides them, polyphenols also include the groups of flavonoids, stilbenes, lignans and phenolic alcohols [24].

Since only phenolic acids and flavonoids are of importance for this thesis, these two classes are described in the following.

Phenolic acids

Phenolic acids are found in almost all parts of the plant, for example in stems, leaves and flowers as well as in the fruits [142–145]. They always consist of one aromatic ring and can be divided into the hydroxybenzoic and the hydroxycinnamic acids (Figure 2.4) [30, 145, 146].

Hydroxybenzoic acids are usually found in very small amounts in edible plants and in higher amounts in black radish, red berries and onions. Important representatives of hydroxybenzoic acids are siringic acid, gallic acid, and 4-hydroxybenzoic acid [30, 145]. **Hydroxycinnamic acids** are more widespread in nature than the hydroxybenzoic acids. They are found in larger amounts in fruits than in vegetables. Among the best known hydroxycinnamic acids are caffeic acid, *p*-coumaric acid, and ferulic acid [145, 146].

Like most of the polyphenols, phenolic acids are responsible for flavor, coloration, and other factors that influence the organoleptic properties of foods [145, 147, 148]. These properties are exploited by the industry, which is why phenolic acids are used, for example, as additives in food [145, 147–152]. They are also

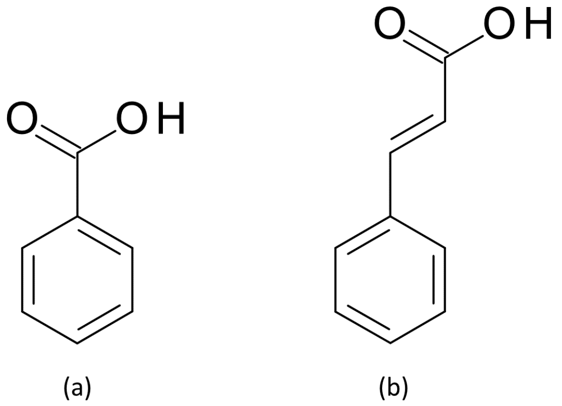

Figure 2.4: Subdivision and classification of phenolic acids ((a) hydroxybenzoic acid, (b) hydroxycinnamic acid).

attributed with health-promoting properties because of their anti-inflammatory and antioxidant activity [145, 153]. However, they are also used, for example, as additives for color retention, against microbial spoilage or protection against oxidation [145, 147–152].

Flavonoids

Flavonoids are mainly responsible for the coloration and protection of the plant (UV protection, antimicrobial, etc.) [154–156]. Since they are known for their biochemical properties and antioxidant effects, they are considered to have health-promoting properties, such as an anticancerogenic effect. For this reason, they are often used in cosmetical, pharmaceutical, nutraceutical and medical applications [157–159]. Flavonoids consist of the basic structure of flavane (Figure 2.5), which is composed of three rings (A-, B- and C-ring), consisting of 15 carbon atoms. Flavonoids themselves can be further divided into six subgroups (Figure 2.5), i.e., flavones, flavanols, flavonols, isoflavones, anthocyanins, and flavanones. The type of flavonoid depends on the oxidation state of the central carbon ring and the degree of unsaturation. The wide variation of subgroups is partly dependent on the structure and degree of hydroxylation, methoxylation, prenylation, and glycosylation [24, 154].

The **flavones**, along with the flavonols, are one of the most important subgroups of flavonoids. Just like the flavonols, they also have a double bond between C2 and C3 and a ketone group at C4 of the C-ring and most substances also own a hydroxyl group at C5 of the A-ring. Flavones occur in parsley and camilla, but also in the peel of citrus fruits. Representatives are luteolin and apigenin [154, 160].

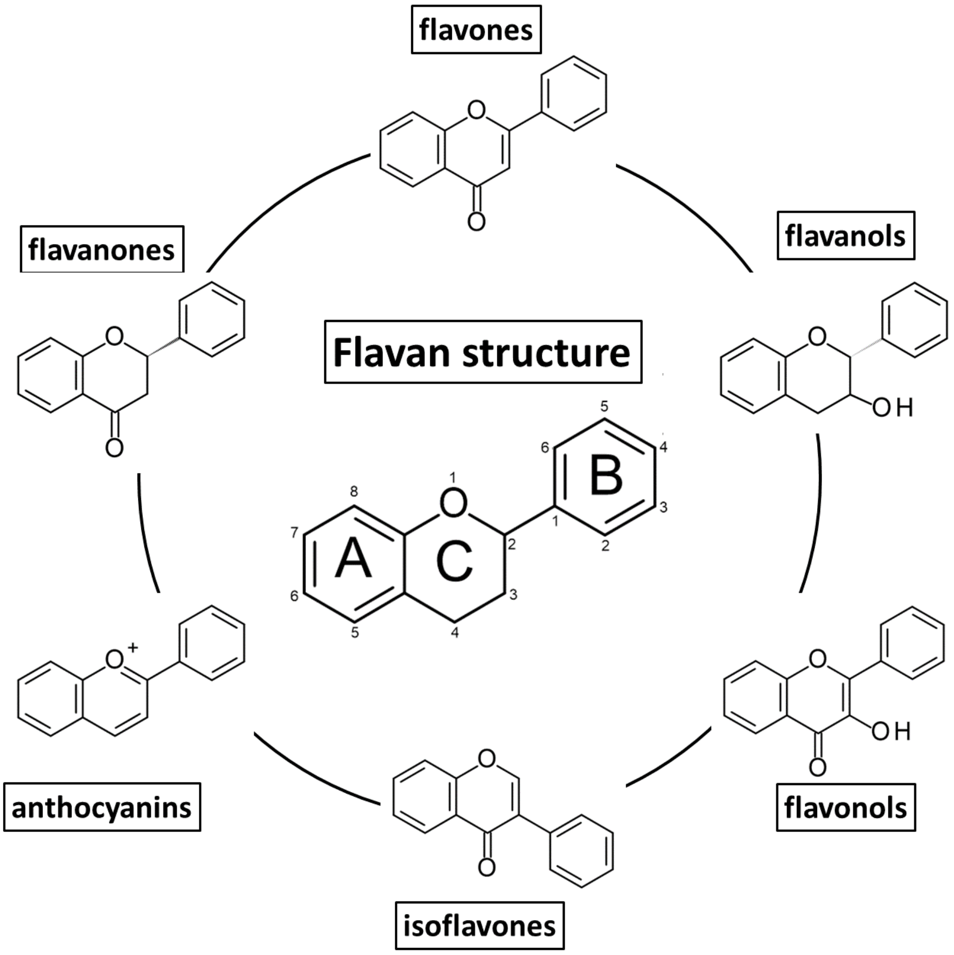

Figure 2.5: Classification and structural properties of flavonoid subgroups.

Flavanols are flavanones without a 4-oxo group and with an additional hydroxyl group at position 3 of the C-ring. They are found, for example, in pears, bananas, and apples [154]. Examples of this subgroup are (-)-epicatechin and (+)-catechin [161, 162].

The **flavonols** are the most widespread and largest subgroup of flavonoids. They own a double bond between position C2 and C3 of the A-ring, a ketone on C4 of the A-ring and a hydroxyl group at C3 of the C-ring, which may also be glycosylated. They are found in grapes, blueberries, onions, apples, and broccoli. Most studied examples of flavonols are quercetin, kaempferol, myricetin, and fisetin [24, 154, 163, 164].

Isoflavones have a low prevalence and are found in legumes and soybeans, but have also been detected on microbes [165–167]. They are known for their estrogenic activity and are therefore also considered as phytoestrogens. Examples of isoflavones are genistein and daidzein [168].

The **anthocyanins** are responsible for the coloration in fruits and flowers, whereby the color strongly depends on the pH-value [154, 169]. Furthermore, they have health-promoting properties and are therefore used as herbal medicines for the treatment of hypertension, fever, liver diseases, dysentery and diarrhea, urinary tract diseases, and colds. However, they also find their application in the food industry, where they are mainly used as colorants [170, 171]. Commonly, they are found in red grapes, black currants, and cranberries. Examples of anthocyanidins are malvidin, delphinidin, and cyanidin [154, 164, 169].

Structurally, **flavanones** are very similar to flavones, with the difference that flavanones lack the double bond on the C-ring, which makes them unsaturated. Like flavones, flavanones are found in citrus fruits such as lemons and oranges. Mostly they are found in the peel and are responsible for the bitter taste. They are also attributed to properties such as lowering blood lipid and cholesterol, and anti-inflammatory properties [154, 164]. Examples of flavanones are hesperetin, naringin, naringenin, and taxifolin.

The group of **chalcones** is structurally very different from the other flavonoids since they do not have a C-ring and thus have an open-chain structure, which is why, depending on the definition, they are only partially counted to the flavonoids. Well-known representatives of this group are phloridzin and phloretin, which are the main secondary plant metabolites in apples [154, 172].

Both phenolic acids and flavonoids are known for their antioxidant activity. The structural properties on which this depends are explained in more detail in the following chapters.

2.3.2 Antioxidant behavior

The antioxidant effect of phenolic compounds depends on their structural properties such as the type and position of the substituents, which is also called structure-activity relationships [173]. A well-known classification of the structural properties of antioxidants are the Bors criteria, established by Wolf Bors in 1990 for flavonoids [174]. A representation of these structural criteria is shown in Figure 2.6.

The first Bors criterion (Bors 1), is defined as the presence of a catechol group on the B-ring, which stabilizes the antioxidant radical. If the molecule has a 2,3-double bond together with a 4-oxo group on the C-ring, this facilitates electron delocalization and is defined as the second Bors criterion (Bors 2). The third Bors criterion (Bors 3) is the presence of a hydroxyl group at C3 and C5 on the A-

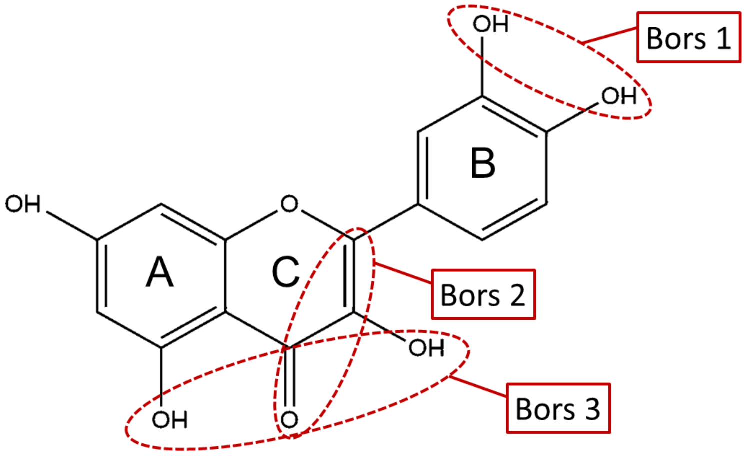

Figure 2.6: Structural representation of Bors criteria (reproduced from Platzer et al. [43, 175]).

and C-rings and a 4-oxo group on the C-ring, which allows electron delocalization via the hydrogen bonds [174].

In addition to the Bors criteria, there are other structural properties that have an influence on the antioxidant effect as for example the number of hydroxyl groups and the presence of methoxy groups or sugar residues and structural orientation [176–184].

The reaction mechanisms and structure-activity relationships of antioxidants are commonly investigated using *in vitro* antioxidant assays, which are based on different reaction mechanisms as described in the following.

2.3.3 In vitro reaction mechanisms of antioxidants

Antioxidants can undergo different reaction mechanisms, which include the hydrogen atom transfer (HAT) and the single electron transfer (SET), further subdivided into single electron transfer followed by a proton transfer (SET-PT) and the sequential proton loss electron transfer (SPLET). The sequences of the different reactions are described below [185].

If an antioxidant reacts with a radical, both mechanisms take place, but due to different reaction kinetics they differ in speed [186–189]. Which reaction proceeds preferentially depends on several factors, including the nature of the radical and the solvent. As an example, in nonpolar solvents, the HAT mechanism is dominant, whereas the SET mechanism occurs mainly in polar solvents. The exception are strongly oxidizing radicals, since these also react in nonpolar solvents by SET. Other factors influencing the preferred mechanism are the chemical structure and the prevailing pH during the reaction [190]. In the following, the two reaction mechanisms are explained in more detail.

In the HAT-based mechanism, the antioxidant (AOH) reacts with a free radical (R·) by donating a hydrogen atom, thus interrupting the oxidative chain reaction (2.14) [187, 189, 191–193],

$$\mathrm{R}\cdot + \mathrm{AOH} \longrightarrow \mathrm{RH} + \mathrm{AO}\cdot. \tag{2.14}$$

Thereby an an antioxidant radical (AO·) [194] is formed and the reaction is influenced by the bond dissociation energy, which is responsible for the binding strength of the hydroxyl groups. The lower the bond dissociation energy, the easier the release of a hydrogen atom and the higher the antioxidant effect [187, 189, 191–193]. According to the work of Zheng et al. [189], the number and position of hydroxyl groups on the B- and C-rings are especially crucial for HAT-based mechanism. Furthermore, the stability of the resulting antioxidant radical is important for the antioxidant effect. The higher the stability, the higher the antioxidant effect, which can be explained by a low electron spin density [187, 189–193].

In SET-PT mechanism, neutralization of a free radical occurs through the donation of an electron from the antioxidant,

$$\mathrm{R}\cdot + \mathrm{AOH} \longrightarrow \mathrm{R}^- + \mathrm{AOH}^+\cdot, \tag{2.15}$$

followed by the transfer of a proton,

$$\mathrm{R}^- + \mathrm{AOH}^+\cdot \longrightarrow \mathrm{RH} + \mathrm{AO}\cdot. \tag{2.16}$$

In the first reaction step (2.15), the ability to donate electrons depends on the possibilities of electron delocalization throughout the molecule, i.e., a high degree of π-delocalization leads to a higher antioxidant activity [187, 189, 191–193]. Another influencing factor is the ionization potential, i.e., the required energy for the release of an electron. The smaller it is, the easier the release of an electron and the higher the antioxidant effect. In the second reaction step (2.16) of the SET-PT, where a proton is transferred, the proton dissociation enthalpy plays a decisive role. Zheng et al. [189] found that the abstraction of a hydrogen atom from the A-ring is much more difficult than from the B- or C-ring. Accordingly, the release of a hydrogen atom from the B- or C-ring plays a crucial role for this step SET-PT [189, 191–193]. In the SET-PT mechanism, the formation and breaking of positively charged flavonoid cation radicals occurs, which are known to be affected by the polarity of the surrounding solvent [189, 191–193]. The radical cations are more stable in the polar solvent, and the delocalization and conjugation of the π-eletrons are stronger. Therefore, the donation of electrons in polar solvents is preferred [189].

During the SPLET mechanism, the reactions take place in reverse order, i.e., a proton is released before an electron. This mechanism occurs preferentially in polar solvents and plays a crucial role there [189, 191–193]. In the first step of the SPLET reaction (2.17), formation of an antioxidant anion (AO^-) occurs,

$$\mathrm{AOH} \longrightarrow \mathrm{AO}^- + \mathrm{H}^+. \tag{2.17}$$

This step is influenced by the proton affinity, if this is low, the antioxidant effect is higher. According to Zheng et al. [189], the formation of an anion is easiest on the B-ring and most difficult on the A-ring. Again, the hydroxyl groups on the B-ring possess the highest influence on the antioxidant behavior [189]. In the second step of the SPLET mechanism (2.18), the formation of a flavonoid radical occurs by donation of an electron,

$$\mathrm{AO}^- + \mathrm{R}\cdot \longrightarrow \mathrm{AO}\cdot + \mathrm{R}^-, \tag{2.18}$$

and R^- further reacts with a hydrogen atom,

$$\mathrm{R}^- + \mathrm{H}^+ \longrightarrow \mathrm{RH}. \tag{2.19}$$

The lower the electron transfer enthalpy, the easier it is to split electrons in Reaction 2.19 and the higher the antioxidant effect. This step also depends particularly on the hydroxyl groups on the B- and C-rings of the flavonoid. The hydroxyl groups on the A-ring are rather not important here [189].

2.4 In vitro determination of antioxidant activity

To determine the effect of antioxidants, there are several *in vitro* assays, such as the ABTS, DPPH, FC, ORAC, total radical trapping antioxidant parameter (TRAP), ferric reducing antioxidant power (FRAP), cupric ion reducing antioxidant capacity (CUPRAC), and N,N-dimethyl-p-phenylenediamine (DMPD). The results of the assays depend on different structural properties based on the structure-activity relationships. In the following, the *in vitro* assays relevant for this thesis, i.e., ABTS, DPPH, FC, and ORAC, are described in more detail.

2,2-Diphenyl-1-picrylhydrazyl (DPPH)

For the DPPH assay (SET-based) a synthetic radical is used, which was first used by Blois et al. [195] and was later adapted in an optimized version of the assay in the work of Brand-Williams et al. [196]. The DPPH radical is colored deep purple and turns colorless when reacting with an antioxidant, which becomes a radical itself [195, 196]. There are different ways of evaluating and measuring the DPPH assay, including endpoint measurements, where the measurement is stopped at a specific point of time. However, there is also the possibility of measuring the assay as a kinetics function over time. Another difference in performance is the number of samples. Often the sample is measured in only one concentration and sometimes in different dilutions [196–199]. There are also different approaches regarding the evaluation of the raw data. In some studies the values are given as half maximal effective concentration (EC_{50}). However, the time to reach the EC_{50} can also be calculated, which is then referred as the time to reach EC_{50} (T_{EC50}). Other possibilities to indicate the result are the stoichiometry, the antiradical power, the reaction rate, and kinetic behavior as well as the radical scavenging activity and Trolox equivalents [196, 199–205]. Therefore, a comparison with the literature is often difficult.

Nevertheless, the DPPH assay is very popular as it provides a rapid way to determine the antioxidant behavior of samples or to study the kinetic profiles [203]. Furthermore, it is an inexpensive method, which can be performed with standard laboratory equipment. It is known from literature that the assay can be used for both polar and non-polar samples, which makes it suitable for a wide range of applications. Compared to other assays, the radical is also fast to use, as it requires no major preparation, can be purchased ready-to-use, and has a high stability [196].

A disadvantage of the assay is that the DPPH radical is a reactive and volatile peroxide involved in lipid peroxidation. Substances that have a high reaction rate react with the DPPH radical in different kinetics, which in turn can lead to a distortion of the final result. Some antioxidants also do not react with the radical at all, which also leads to underestimation of the antioxidant effect [206]. It should also be noted that different substances react at different rates and also at a different stoichiometry. These factors are not taken into account in simple variants of the measurement method and evaluation methodologies, such as endpoint measurements [196]. Another disadvantage is that the assay is only conditionally suitable for hydrophobic substances, since the DPPH radical is only soluble in polar solvents [207].

2,2'-Azobis-(2-methylpropionamidine)-dihydrochloride (ABTS)

The ABTS assay (SET-based), also called trolox equivalent antioxidant capacity (TEAC) assay was first developed by Miller et al. [208] and is based on a color change of the ABTS radical. In 1996, the method was revised and adapted based on several critiques. In the new version of the assay, the ABTS radical is oxidized to a stable complex with manganese dioxide before the measurement in order to prevent underestimantion of the antioxidant effect [209]. The method was subsequently adapted again by Re et al. [40] to enable the measurement of water-insoluble substances. For this purpose, by the addition of potassium peroxide, ABTS cations are generated, which have a green-blue color. Just as in the DPPH assay, decolorization occurs when the radicals react with an antioxidant, which can be determined spectrophotometrically at a wavelength of 734 nm [40].

The ABTS assay is an inexpensive methods for the determination of antioxidant activity, which can be easily performed with conventional laboratory equipment [206]. By optimizing the assay, Re et al. [40] were able to measure lipophilic substances, making the assay suitable for a variety of applications [40, 207]. Another advantage is the possible automatization of the method, which allows to measure a larger number of samples in a sequence and also to achieve high reproducibility [210–212]. Possible interfering factors such as pH and sample color play a minor role in this test, which is another advantage in contrast to other measurement methods [209, 213].

However, this measurement method also has some disadvantages, such as the sample preparation, which must be carried out 12-16 h before the measurement and prevents spontaneous measurements [40]. In addition, the radical, just like the DPPH radical, is a synthetic radical, which resembles a natural radical but behaves differently. Therefore, a comparison with the desired applications is only possible to a limited extent [214]. In addition, there are factors such as the sample matrix that can distort the results and lead to either an overestimation or underestimation of the result [215].

Folin Ciocalteu (FC)

Another method to determine the antioxidant behavior is the FC assay (SET-based) [42, 206, 216–218]. Originally, the assay was developed for the quantification and detection of tyrosine [219]. Later, it was further developed and modified by Singelton and Rossi [220] to determine the total polyphenol content in wine. Just like the DPPH and ABTS assays, this assay is based on a color reaction. It is known that the FC reagent is responsible for the color reaction, but its exact composition is unknown. It is believed to be a mixture of heteropolyacid, phosphormolybdenum, and phosphotungstic acid, which are responsible for the color change from yellow to blue [206, 220]. The color change can be determined spectrophotometrically at a wavelength of 765 nm and the result is often correlated to gallic acid equivalents [220].

Despite the unknown composition of this FC reagent, the FC assay is the most popular method for the determination of total polpyhenol content. This method is also simple and cheap and gives well reproducible results [221]. The FC reagent is available in a ready-to-use condition and has a high storage stability [220].

A disadvantage of the assay is that there are many susbtances that could interfere with FC reagent and thus distort the results. These include aromatic amines, organic acids, and metals, as well as many other substances [220, 222]. Furthermore, it is discussed in the literature whether the assay actually measures the total polyphenol content or the antioxidant activity. Although, according to the current state of the art, the FC assay is classified as an antioxidant measurement method, it is still used for the determination of total polyphenol content and the results are published using different units. This makes a comparison with measurement data from the literature difficult and sometimes even impossible [223–225].

Oxygen radical absorbance capacity (ORAC)

The ORAC assay (HAT-based) is based on fluorescence measurement and was originally decribed by Cutler and Cao [226]. The first version of the assay was performed using B-phycoerythrin, which is derived from a red algae and varies from batch to batch. This led to poor reproducibility of measurement results. Another disadvantage was that photobleaching, i.e., loss of fluorescence, may occur during measurement in the microplate reader. Moreover, the B-phycoerythrin may lose its fluorescence even without the presence of a radical generator, thus distorting the results [206, 227]. Because of the many drawbacks, the method was adapted by Ou et al. [221] by replacing the B-phycoerythrin with another fluorescent substance, i.e., fluorescein. It is a synthetic substance that ensures stable and consistent quality [206, 221, 228]. When performing the measurements, the samples are mixed with a fluorescein-buffer solution and are placed in a 96-well plate. After incubation, 2,2'-azobis-(2-methylpropionamidine)-dihydrochloride (AAPH) is added, the reaction starts and proceeds at a pH of 7.4, which is adjustied by a buffer. The reaction of fluorescein with the AAPH causes a decrease in fluorescence, which can be detected at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. If antioxidants are present, the fluorescein reacts preferentially with them, which initially prevents the fluorescence decrease. Once the antioxidants are depleted, the fluorescein reacts with the AAPH radical. Therefore, in the presence of antioxidants, there is a delayed decrease in fluorescence compared to the blank. To determine the antioxidant activity, the area under the curve of the obtained trace is determined and adjusted with the area of the blank. Often, the result is expressed in trolox equivalents rather than the area under the curve [206].

By measuring the ORAC assay in a micro-plate reader it is possible to measure many samples in a short time, which can also be done automatically (automatic micro-plate reader). The ORAC assay is particularly suitable for samples with a complex matrix, which is why it is often used in the food industry [37, 206, 226, 229]. In addition, the ORAC assay in a modified version is also suitable for the measurement of lipophilic samples. However, it should be mentioned here, that the assay is less sensitive in these media [206, 230]. In addition, the assay is suitable for measuring antioxidants with a high or a low lag phase [206, 226]. Another advantage is that it measures time and degree of inhibition in addition to antioxidant activity. The evaluation methods take into account factors such as time lag and different initial rates, making the assay suitable for complex samples [206].

Disadvantages of the ORAC assay include thermally induced radical formation from AAPH, which can be responsible for variations in results [227]. Another point of criticism is the time-consuming sample preparation and execution of the method, which does not apply to fully automated method execution. Since these fully automated devices are not part of the standard equipment of conventional laboratories and are associated with increased costs, the assay is usually performed manually [231, 232].

Shortcomings and disadvantages of in vitro antioxidant assays

As describted above, *in vitro* methods of the determination of antioxidant activity have many advantages and disadvantages and are therefore repeatedly criticized [218]. They are based on different reaction mechanisms and can be influenced by sevaral factors such as temperature, measurement duration, and pH leading to over- or underestimation of the antioxidant activity [206, 218, 233]. Moreover, different measurement and evaluation methods are frequently reported in the literature and no accepted standard methods are described, which makes it difficult or even impossible to compare measurement results [218, 234]. It is also important to understand that the assays are based exclusively on the chemical reactions that take place *in vitro* and these have no similarities to biological systems [206]. Both high and low correlations between *in vitro* antioxidant assays have been reported in the literature [206, 218, 234–237]. For example, a high correlation between total polyphenol content, measured by the FC assay, and various *in vitro* antioxidant assays, such as the ABTS and DPPH assay, has been reported [206, 235–237]. Furthermore, in the field of biomarker use, a relationship with *in vitro* assays could be established and thus conclusions could be drawn in relation to practical requirements [218, 238–245]. It is also known from the literature that both the FC and ORAC assays can be applied to the inhibition of FA autoxidation in a food system [206, 237, 246, 247]. However, it should be noted that both assays are performed in a controlled and homogeneous medium and these conditions do not exist in the application medium. Furthermore, there are external influences on the sample, such as the presence of metal ions and the influence of light and heat, which change the result. Therefore, the behavior of antioxidants in the *in vitro* assays may differ from the behavior in food. Further research is needed to transfer the results from *in vitro* assays to real application systems and results from such assays should be used only as a first indication [206].

3 Aim and content of the thesis

A variety of different *in vitro* assays for the determination of antioxidant activity and total polyphenol content have been developed and each of them has its advantages and disadvantages. Since the assays are not performed in a standardized way and there are no uniform methods of measurement and evaluation, a comparison between different studies is often difficult or impossible. Nevertheless, the assays are very popular, which is partly due to the easy and cheap executability and the fact that standard laboratory equipment is sufficient for the measurement. Although there are many literature reports on the different assays, it is still unclear which assay depends on which structural properties and how they correlate. Furthermore, there are hardly any comparisons in the literature between the results of the *in vitro* assays with the effect in the application medium. Accordingly, it is unclear which assay is best suited for which application. Also, while the assays are popular in the food industry, their suitability to predict the antioxidant activity in technical applications such as lubricants, is unclear. Methods used to determine the oxidative stability of vegetable oils include for example the rancimat method and the P-DSC measurement. These require either a large amount of sample, have long measurement times or cannot be performed with standard laboratory equipment. As the *in vitro* antioxidant assays require a much shorter measurement time and sample amount and can be performed in any standard laboratory, their use would be particularly beneficial for the lubricant industry.

Thus, the aim of this thesis was to deepen the knowledge about *in vitro* assays for the determination of antioxidant activity and to learn more about the structure-activity relationships and the reaction mechanisms. In addition, the adaptation of measurement and evaluation methods should lead to a standardization and thus to a higher comparability. Furthermore, it should be clarified, which of the assays is suitable for the prediction of the antioxidative effect of different phenolic substances in rapeseed oil and whether it is possible to replace the standard methods by *in vitro* antioxidative assays. The results of the thesis should also allow to evaluate the suitability of plant ingredients as alternative antioxidants for vegetable oil-based lubricants.

For this purpose, different SET- (ABTS, DPPH, FC) and HAT-based (ORAC) assays as well as two conventional measurement methods for the determination of oxidative stability of oils (rancimat method and P-DSC) were used for measuring the antioxidant potential of different phenolic substances. The structure-activity relationshisp were investigated more in detail to determine structural features of phenolic compounds that correlate with a high antioxidant activity in the assays and in oil. It was analyzed, whether the use of an *in vitro* assay already predicts the effect on the oil and therefore provides a suitable method to replace costly oil tests.

4 Results

The results chapter consists of the following peer-reviewed publications:

- Platzer M., Kiese S., Herfellner T., Schweiggert-Weisz U., Miesbauer O., Eisner P., Common trends and differences in antioxidant activity analysis of phenolic substances using single electron transfer based assays, *Molecules* **26**, 1224 (2021)
- Platzer M., Kiese S., Herfellner T., Schweiggert-Weisz U., Eisner P., How does the phenol structure influence the results of the Folin-Ciocalteu assay?, *Antioxidants* **10**, 811 (2021)
- Platzer M., Kiese S., Tybussek T., Herfellner T., Schneider, F., Schweiggert-Weisz U., Eisner P., Radical scavenging mechanisms of phenolic compounds: A quantitative structure-property realtionship (QSPR) study, *Frontiers in Nutrition* **9**, 663 (2022)
- Platzer M., Kiese S.,Asam T., Schneider F., Tybussek T., Herfellner, T., Schweiggert-Weisz U., Eisner P., Quantitative structure-property relationship (QSPR) of plant phenolic compounds in rapeseed oil and comparison of antioxidant measurement methods, *Processes* **10**, 1281 (2022)

4.1 Common trends and differences in antioxidant activity analysis of phenolic substances using single electron transfer based assays [1]

Summary

There are numerous methods of determining the antioxidant activity, each having unique characteristics and limitations and being performed using different measurement and evaluation methods, which often makes comparison of results difficult or even impossible. For this reason, 24 phenolic compounds of different subclasses (phenolic acids, flavonols, flavanones, dihydrochalcones, and flavanols) were measured in two SET-based antioxidant assays, i.e., ABTS and DPPH, using the same methodology.

The highest influence on the results of the two assays was attributed to the correct implementation of the measurement and evaluation methods. The substances showed different kinetic behaviors, resulting in different reaction times and making the measurement time the decisive parameter. Accordingly, some substances, e.g. phloretin, needed several hours to reach the endpoint of reaction, while others, such as 4-hydroxybenzoic acid, finished in a few minutes. By using second-order reaction kinetics, it was possible to calculate the final values of the reaction and shorten the measurement time of the assays from above 24 h down to 30 min. Furthermore, the study revealed that measuring each sample as a dilution series is necessary to avoid underestimation of the antioxidant effect of the samples.

In addition to the influence of the methodology, the study also intensively dealt with the structure-activity relationships between the phenolic substances and the radicals used in the two assays. Although, the same measurement and evaluation methods were used, different trends emerged for the two assays and they were found to depend on different structural properties. The results of the DPPH assay showed a strong dependence on the Bors criteria, especially on Bors 1 and 3, as well as on the number of hydroxyl groups. Accordingly, the subgroups of flavonols and flavanols obtained the highest stoichiometry and therefore, the highest antioxidant activities. It was also found that the DPPH assay is not suitable to measure extracts rich in dihydrochalcones or flavanones, since these

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[1] Platzer M., Kiese S., Herfellner T., Schweiggert-Weisz U., Miesbauer O., Eisner P., Common trends and differences in antioxidant activity analysis of phenolic substances using single electron transfer based assays, *Molecules* **26**, 1224 (2021)

substances partially did not react with the DPPH radical. Therefore, the assay does not seem suitable for extracts containing large amounts of these compounds. Otherwise there is an underestimation of the antioxidant effect. In contrast, the results of the ABTS assay neither depended on the Bors criteria nor the number of hydroxyl groups, and the structure-activity relationships of this assay could not be clarified conclusively.

In summary, different trends and structure-activity relationships were found for the two different SET-based assays, and the correlation to the antioxidant behavior in oils is still to be analyzed. Furthermore, to use these assays for the measurement of natural extracts, other influential parameters such as pH, solvents, and the content of carbohydrates and protein should be further investigated.

Author contributions:

- Melanie Platzer: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing-original draft preparation and visualization
- Sandra Kiese: conceptualization, methodology, resources, writing-review and editing, supervision and funding acquisition
- Thomas Herfellner: resources, writing-review and editing, supervision and funding acquisition
- Ute Schweiggert-Weisz: writing-review and editing and supervision
- Oliver Miesbauer: methodology, validation and writing-review and editing
- Peter Eisner: validation and writing-review and editing and supervision

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Article

Common Trends and Differences in Antioxidant Activity Analysis of Phenolic Substances Using Single Electron Transfer Based Assays

Melanie Platzer [1,2,*], Sandra Kiese [2,3], Thomas Herfellner [2], Ute Schweiggert-Weisz [2,4], Oliver Miesbauer [3] and Peter Eisner [1,2]

[1] ZIEL-Institute for Food & Health, TUM School of Life Sciences Weihenstephan, Technical University of Munich, Weihenstephaner Berg 1, 85354 Freising, Germany; peter.eisner@ivv.fraunhofer.de
[2] Fraunhofer Institute for Process Engineering and Packaging IVV, Giggenhauser Str. 35, 85354 Freising, Germany; sandra.kiese@ivv.fraunhofer.de (S.K.); thomas.herfellner@ivv.fraunhofer.de (T.H.); ute.weisz@ivv.fraunhofer.de (U.S.-W.)
[3] Chair of Food Packaging Technology, TUM School of Life Sciences Weihenstephan, Technical University of Munich, Weihenstephaner Steig 22, 85354 Freising, Germany; oliver.miesbauer@tum.de
[4] Chair of Food Science, Institute for Nutritional and Food Sciences, University of Bonn, Meckenheimer Allee 166a, 53113 Bonn, Germany
* Correspondence: melanie.platzer@ivv.fraunhofer.de

Abstract: Numerous assays were developed to measure the antioxidant activity, but each has limitations and the results obtained by different methods are not always comparable. Popular examples are the DPPH and ABTS assay. Our aim was to study similarities and differences of these two assay regarding the measured antioxidant potentials of 24 phenolic compounds using the same measurement and evaluation methods. This should allow conclusions to be drawn as to whether one of the assays is more suitable for measuring specific subgroups like phenolic acids, flavonols, flavanones, dihydrochalcones or flavanols. The assays showed common trends for the mean values of most of the subgroups. Some dihydrochalcones and flavanones did not react with the DPPH radical in contrast to the ABTS radical, leading to significant differences. Therefore, to determine the antioxidant potential of dihydrochalcone or flavanone-rich extracts, the ABTS assay should be preferred. We found that the results of the flavonoids in the DPPH assay were dependent on the Bors criteria, whereas the structure–activity relationship in the ABTS assay was not clear. For the phenolic acids, the results in the ABTS assay were only high for pyrogallol structures, while the DPPH assay was mainly determined by the number of OH groups.

Keywords: antioxidant capacity; flavonoids; phenolic acids; reaction kinetics; stoichiometry

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Citation: Platzer, M.; Kiese, S.; Herfellner, T.; Schweiggert-Weisz, U.; Miesbauer, O.; Eisner, P. Common Trends and Differences in Antioxidant Activity Analysis of Phenolic Substances Using Single Electron Transfer Based Assays. *Molecules* **2021**, *26*, 1244. https://doi.org/10.3390/molecules26051244

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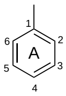
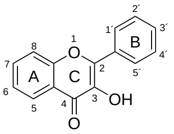
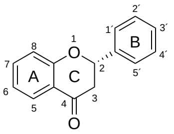
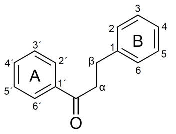
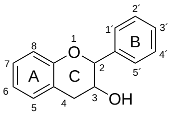
1. Introduction

Plant-based antioxidants are known for their ability to limit radical reactions by transferring hydrogen atoms or electrons and to interrupt the chain reactions of oxidative degradation [1–5]. Among the most important groups of plant-based antioxidants are phenolic compounds, which have one or more aromatic rings with one or more hydroxy groups. They are common plant secondary metabolites and are divided into several major families according to their chemical structure, including the flavonoids and phenolic acids [6]. The phenolic compounds investigated in this article are listed in Table 1.

Table 1. Summary of the phenolic compounds, reference standards, sample codes and corresponding side groups.

Group	Reference Standard	Sample Code	Side Group					
phenolic acids			**1**	**3**	**4**	**5**		
	caffeic acids	CAA	(CH)$_2$COOH	OH	OH	H		
	3,4-dihydroxybenzoic acid	DBA	COOH	OH	OH	H		
	ferulic acid	FEA	(CH)$_2$COOH	OH	OCH$_3$	H		
	gallic acid	GAA	COOH	OH	OH	OH		
	4-hydroxybenzoic acid	HBA	COOH	H	OH	H		
	p-coumaric aicid	PCA	(CH)$_2$COOH	H	OH	H		
	sinapic acid	SIA	(CH)$_2$COOH	OCH$_3$	OH	OCH$_3$		
	siringic acid	SRA	COOH	OCH$_3$	OH	OCH$_3$		
flavonols			**2′**	**3′**	**4′**	**5′**	**3**	**5**	**7**
	kaempferol	KAE	H	H	OH	H	OH	OH	OH
	myricetin	MYR	H	OH	OH	OH	OH	OH	OH
	morin	MOR	OH	H	OH	H	OH	OH	OH
	quercetin-3-D-galactoside	QGA3	H	OH	OH	H	Glc	OH	OH
	quercetin-3-D-glucoside	QGU3	H	OH	OH	H	Gal	OH	OH
	quercetin	QUR	H	OH	OH	H	OH	OH	OH
flavanones			**3′**	**4′**	**5**	**7**		
	hesperetin	HES	OH	OCH$_3$	OH	OH		
	narirutin	NAR	H	OH	OH	2 Glc		
	naringenin	NAN	H	OH	OH	OH		
	taxifolin	TAF	OH	OH	OH	OH		
dihydrochalcones			**4**	**2′**	**4′**	**6′**		
	phloridzin	PHD	OH	OH	OH	Glc		
	phloretin	PHT	OH	OH	OH	OH		
flavanols			**3′**	**4′**	**3**	**4**	**5**	**7**
	(+)-catechin	CAT	OH	OH	OH	H	OH	OH
	(−)-epicatechin	EPC	OH	OH	OH	H	OH	OH
	proanthocyanidin B1	PCB1	OH	OH	OH	CAT	OH	OH
	proanthocyanidin B2	PCB2	OH	OH	OH	EPC	OH	OH

Three criteria for the structure-activity relationship (SAR) of antioxidant compounds have been defined by Bors [7]:

- Bors 1—due to hydrogen bonding, the presence of a catechol group on the B-ring leads to a high stability of the antioxidant radical (AO·);
- Bors 2—a 2,3 double bond in combination with a 4-oxo group on the C-ring facilitates electron delocalization;
- Bors 3—the presence of OH groups at position 3 and 5 in combination with the 4-oxo group enables electron delocalization via hydrogen bonds.

The presence of a pyrogallol structure on the B-ring can also increase the antioxidant activity [8]. Molecules that fulfil all of the Bors′ criteria, such as quercetin (QUR) and myricetin (MYR), should therefore achieve the most efficient electron delocalization and accordingly should possess the highest antioxidant activity [7,9]. Attempts to correlate chemical structures with antioxidant activity are usually based on the analysis of natural phenolic compounds and extracts using various antioxidant assays [3]. The most widely used methods are based on oxygen radical absorbance capacity (ORAC) [10], 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) [11] and 2,2-diphenyl-1-picrylhydrazyl (DPPH) [12] assays. The underlying chemistry involves either hydrogen atom transfer (HAT) or single electron transfer (SET) as shown in Figure 1 [3].

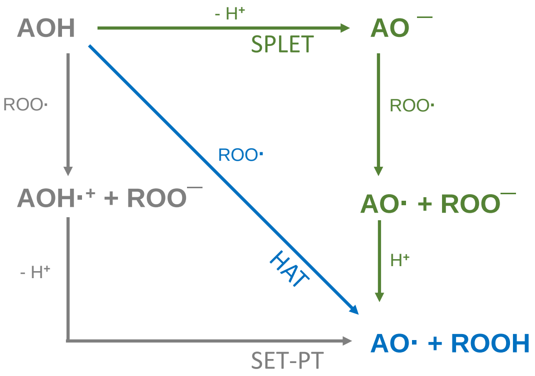


Figure 1. Schematic representation of HAT, single electron transfer followed by a proton transfer (SET-PT) and sequential proton loss electron transfer (SPLET) antioxidant reaction mechanisms (reproduced from Shang et al. (2009) [13] with some modifications).

HAT assays (such as ORAC) measure the ability of an antioxidant (AOH) to inactivate a free radical (ROO·) by releasing a hydrogen atom [3,4,14]. In contrast, assays that are dominated by SET-based reaction mechanisms, such as ABTS and DPPH, measure the release of an electron to the (ROO·), converting it into an anion (ROO^-) [4,15,16]. The latter causes reversible deprotonation and a color decrease in the solution, which not only indicates the reaction endpoint but simultaneously reports the concentration of the antioxidant [3,17]. SET mechanisms can be subdivided into SET-PT and SPLET. The two-stage SET-PT mechanism strongly depends on the ionization potential and proton dissociation energy. Antioxidants, which are easily ionized and deprotonated, are highly reactive. In contrast, the SPLET mechanism involves the initial loss of a proton from the antioxidant followed by anion transfer to the radical, which then reacts with the proton. This is influenced by the proton affinity and electron transfer enthalpy. SET-PT and SPLET are in thermodynamic equilibrium with each other (and also with the HAT mechanism), because the reactants and end products are identical. However, the reaction rate in each assay depends on different physical processes [4,15,16]. The dominant reaction depends on the pH and solvent [16], resulting in different SARs for each assay. The ABTS radical preferably reacts via the SPLET mechanism in aqueous solutions, whereas the DPPH radical preferably reacts via the SPLET mechanism in solvents such as ethanol and methanol [18,19]. Some authors have theoretically investigated the reaction mechanisms and classified the antioxidant effect of substances via the SPLET mechanism on the basis of electron transfer energy and proton affinity, but the dependence of these effects on the pH and solvent makes it difficult to make accurate predictions [20–22].

Each of these assays has unique properties and limitations, making it difficult to compare results generated using different methods—for example, measurement at a fixed time or kinetic, measurement of different concentrations or only one concentration, and the use of different solvents [23–26]. Furthermore, the outcome of the assays can be evaluated in different ways, resulting in metrics, such as half maximal effective concentration (EC_{50}), time to reach the EC_{50} ($T_{EC_{50}}$), antiradical efficiency, antiradical power (ARP), stoichiometry, kinetic behavior and rate constant, radical scavenging activity, or trolox equivalent antioxidant capacity (TEAC) [23,24,27–32]. Although there is a large number of publications on the assays and their limitations, little is found on the most suitable use of the assays for different types and applications of extracts.

Our aim was to study whether the DPPH or the ABTS assay should be preferred to analyze the antioxidant potential of extracts rich in different phenolic components, or whether they lead to similar results. Therefore, we investigated the SAR of 24 different phenolic compounds by comparing two SET assays (ABTS and DPPH). The phenolic compounds represented the phenolic acids, flavonols, flavanones, dihydrochalcones, and flavanols, allowing us to compare the antioxidant properties of different groups as well as individual substances within a group and thus determine structural features correlating with high antioxidant activity. We used the same measurement as well as evaluation methods in our study. To ensure that the reactions were complete and the reported values are not underestimated, all measurements were recorded as a function of time. Furthermore, second order kinetic equations were used to verify the endpoint values. EC_{50}, ARP and stoichiometry values are interconvertible without affecting the outcome, so all results are presented herein as stoichiometry values [29].

2. Materials and Methods

Chemicals (antioxidant reference standards) were obtained from MilliporeSigma (Steinheim, Germany): caffeic acid (CAA), (+)-catechin (CAT), 3,4-dihydroxybenzoic acid (DBA), (−)-epicatechin (EPC), ferulic acid (FEA), gallic acid (GAA), 4-hydroxybenzoic acid (HBA), hesperetin (HES), kaempferol (KAE), morin (MOR), myricetin (MYR), naringenin (NAN), p-coumaric acid (PCA), proanthocyanidin B1 (PCB1), proanthocyanidin B2 (PCB2), phloridzin (PHD), phloretin (PHT), quercetin-3-D-galactosides (QGA3), quercetin-3-D-glucosides (QGU3), quercetin QUR, sinapic acid (SIA), siringic acid (SRA) and taxifolin (TAF). These are shown in Table 1. We also obtained DPPH radical, ABTS diammonium salt and potassium persulfate from the same supplier. The narirutin (NAR) reference standard was purchased from J&K Scientific (Marbach am Neckar, Germany) and is also shown in Table 1. Stock solutions were prepared by dissolving the reference standards in absolute ethanol (analytical grade), taking into account the purity, and diluting them in seven steps (0.075–1 mM) for the measurements.

The antioxidant activity was measured using the DPPH [23] and ABTS [11] methods as previously described, with slight modifications. The stock solution for the DPPH assay was prepared by dissolving 24 mg of the radical in 100 mL absolute ethanol. The working solution was prepared diluting the stock solution 1:10. We mixed 50 µL of the sample (reference standard dilution or ethanol blank) with 1950 µL of the working solution for each measurement, and the absorbance was determined by spectrophotometry at 515 nm [23]. The stock solution for the ABTS assay was prepared by dissolving 6.62 mg potassium persulfate and 38.4 µg ABTS diammonium salt in 10 mL demineralized water. This solution was incubated in the dark for 12–16 h and then the working solution was prepared by diluting 1:100 with demineralized water. We mixed 10 µL of each sample (reference standard dilution or ethanol blank) with 990 µL of the working solution for each measurement, and the absorbance was determined by spectrophotometry at 734 nm using a Specord 210 plus spectrophotometer (Analytik Jena, Jena, Germany) [11]. In both assays the absorption was measured as a function of time and the color decrease of the respective radical (DPPH or ABTS) was detected. All measurements were performed in triplicate. The decrease of absorbance in percent was then used to calculate the decrease of initial radical concentration in percent. This corresponds to the amount of radical reduced in mM and was plotted as a function of time. The reaction mechanism is explained below using the DPPH radical, but the principle is the same in the ABTS assay. As mentioned above, all HAT- and SET-based mechanisms lead to identical products (see Figure 1) and therefore can be summarized to Equation (1), where the AOH reacts with DPPH· to form the intermediate AO·

$$\mathrm{AOH} + \alpha\,\mathrm{DPPH}\cdot \longrightarrow \mathrm{DPPH{-}H} + \mathrm{AO}\cdot, \tag{1}$$

which reacts with DPPH· in a second step to yield DPPH-AO Equation (2)

$$\mathrm{AO}\cdot + \beta\,\mathrm{DPPH}\cdot \longrightarrow \mathrm{DPPH{-}AO}, \tag{2}$$

where α and β are stoichiometric coefficients [23,29]. Since only the color decrease of the DPPH radical (DPPH·) is measured and it is not possible to distinguish in which of the

Equations (1) or (2) the radical reacts, only a total stoichiometry (α + β) can be determined with this method. Furthermore, as shown in Equation (3):

$$\mathrm{AO\cdot} + \mathrm{AO\cdot} \longrightarrow \mathrm{AO{-}AO}. \tag{3}$$

Since this reaction does not cause any color decrease in the assay, it is neglected for the following considerations. The reaction of an antioxidant with the DPPH and ABTS reagents follows second-order kinetics [28,29]. Therefore, the changing concentrations of AOH, AO· and DPPH· over time are given as shown in Equations (4)–(6):

$$\frac{\mathrm{d[AOH]}}{\mathrm{d}t} = -k_1[\mathrm{AOH}][\mathrm{DPPH\cdot}], \tag{4}$$

$$\frac{\mathrm{d[AO\cdot]}}{\mathrm{d}t} = k_1[\mathrm{AOH}][\mathrm{DPPH\cdot}] - k_2[\mathrm{AO\cdot}][\mathrm{DPPH\cdot}], \tag{5}$$

$$\frac{\mathrm{d[DPPH\cdot]}}{\mathrm{d}t} = -\alpha k_1[\mathrm{AOH}][\mathrm{DPPH\cdot}] - \beta k_2[\mathrm{AO\cdot}][\mathrm{DPPH\cdot}], \tag{6}$$

where k_1 and k_2 are the reaction rate constants. The initial concentrations $[\mathrm{AOH}]_0$ and $[\mathrm{DPPH\cdot}]_0$ are assumed to be positive, whereas $[\mathrm{DPPH\text{-}H}]_0$ and $[\mathrm{AO\cdot}]_0$ are set to zero. In our experiments, DPPH· was available in excess and accordingly, we assume that its concentration $[\mathrm{DPPH\cdot}]$ on the right side of Equations (4)–(6) is constant. In this case, the reactions follow pseudo-first order kinetics [33] as shown in Equations (7)–(9):

$$\frac{\mathrm{d[AOH]}}{\mathrm{d}t} = -\tilde{k}_1[\mathrm{AOH}], \tag{7}$$

$$\frac{\mathrm{d[AO\cdot]}}{\mathrm{d}t} = \tilde{k}_1[\mathrm{AOH}] - \tilde{k}_2[\mathrm{AO\cdot}], \tag{8}$$

$$\frac{\mathrm{d[DPPH\cdot]}}{\mathrm{d}t} = -\alpha\tilde{k}_1[\mathrm{AOH}] - \beta\tilde{k}_2[\mathrm{AO\cdot}], \tag{9}$$

where the reaction rate constants are defined as $\tilde{k}_1 = k_1[\mathrm{DPPH\cdot}]_0$ and $\tilde{k}_2 = k_2[\mathrm{DPPH\cdot}]_0$. Equation (7) is a first-order linear homogenous differential equation and its analytical solution is given by

$$[\mathrm{AOH}]_t = [\mathrm{AOH}]_0 \exp(-\tilde{k}_1 t). \tag{10}$$

This expression is substituted in Equation (8), which is a first-order linear inhomogeneous differential equation. Its solution is obtained as the sum of the general solution of the corresponding homogeneous equation and a particular solution of the inhomogeneous equation, which is determined by an exponential ansatz as well. Considering the initial condition $[\mathrm{AO\cdot}]_0 = 0$ gives

$$[\mathrm{AO\cdot}]_t = \frac{[\mathrm{AOH}]_0\tilde{k}_1}{\tilde{k}_2 - \tilde{k}_1}\left(\exp(-\tilde{k}_1 t) - \exp(-\tilde{k}_2 t)\right). \tag{11}$$

Substituting $[\mathrm{AOH}]_t$ and $[\mathrm{AO\cdot}]_t$ in Equation (9) and integrating over time leads to the expression for $[\mathrm{DPPH}]_t$, allowing to calculate the amount of DPPH· per volume, which is consumed up to time t, as shown in Equation (12):

$$\begin{aligned} \Delta[\mathrm{DPPH\cdot}]_t &= [\mathrm{DPPH\cdot}]_0 - [\mathrm{DPPH\cdot}]_t \\ &= [\mathrm{AOH}]_0\left(\alpha + \beta + \frac{\alpha\tilde{k}_1 - (\alpha+\beta)\tilde{k}_2}{\tilde{k}_2 - \tilde{k}_1}\exp\left(-\tilde{k}_1 t\right) + \frac{\beta\tilde{k}_1}{\tilde{k}_2 - \tilde{k}_1}\exp\left(-\tilde{k}_2 t\right)\right). \end{aligned} \tag{12}$$

The steady-state values are the asymptotic values of Equation (12) for $t \to \infty$. We adapted this model function to the concentration of reduced radicals, allowing the stationary endpoint of the kinetics to be determined even if the reaction did not reach completion within the measurement time. Assuming that the AOH had completely reacted with the radicals by the reaction endpoint, the measured amount of reduced DPPH and ABTS was plotted as a function of the AOH concentration initially used ($[\mathrm{AOH}]_0$). When plotting the concentration of reduced DPPH ($\Delta[\mathrm{DPPH}\cdot]_t = [\mathrm{DPPH}\cdot]_0 - [\mathrm{DPPH}\cdot]_t$), the positive slope of the linear regression directly indicates the total stoichiometry of the number of DPPH radicals, needed to oxidize the complete AOH in all subreactions (steady state). As shown in Figure 2 as a schematic example, the last two points are in the saturation range, since all DPPH radicals have already been consumed here. In order not to underestimate the stoichiometry, only the concentrations below this range were used for the linear regression. Furthermore, the linear regression equation can be used to calculate the amount of reduced DPPH for higher AOH concentrations, which cannot be determined experimentally.

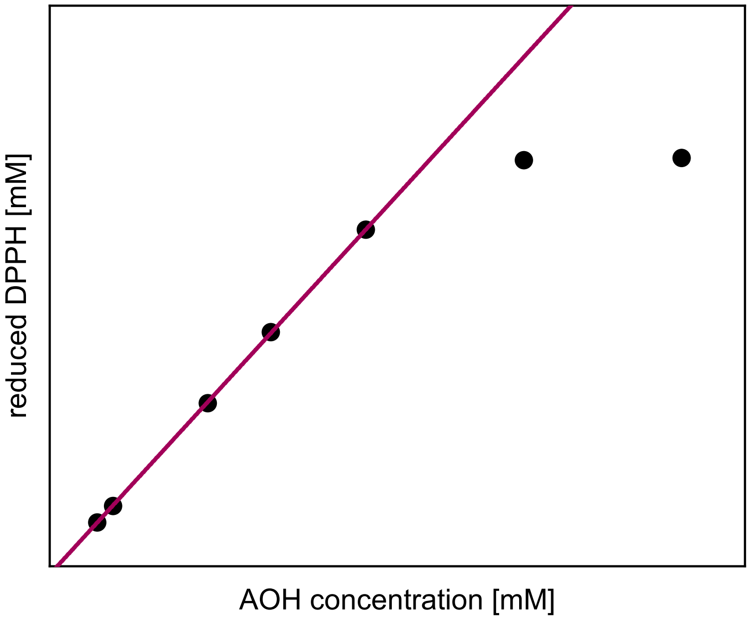


Figure 2. Schematic example of the linear regression for the evaluation of the total stoichiometry of all subreactions.

To determine the stoichiometry values after 5 and 30 min, the fit values after 5 and 30 min were evaluated in the same manner as the steady state stoichiometry. The kinetic behavior of each substance was assigned to one of three groups (fast, medium and slow) as previously described [23]. The fast substances reached steady state within 5 min and the medium ones within 30 min. All substances that needed more than 30 min to reach steady state were assigned to the slow kinetic behavior group. The slower the reaction rate, the more complex the reaction, as previously shown using butylated hydroxytoluene [30].

Statistical evaluation was carried out by one-way analysis of variance (ANOVA) with all significant decimal places using Sigma Plot (Systat Software, San Jose, CA, USA), corresponding to an unpaired *t*-test. If there was a significant difference, an additional pairwise test was performed using the Holm–Šidák method. The significance level for both tests was 0.05.

3. Results and Discussion

The antioxidant activities of 24 polyphenolic reference standards representing five different groups of compounds were determined using DPPH and ABTS assays. The concentration of reduced radicals was then plotted as a function of time. TAF, DBA and CAA are compared in Figure 3 as examples of slow, medium and fast kinetics, showing that only CAA reached the final value after 20 min.

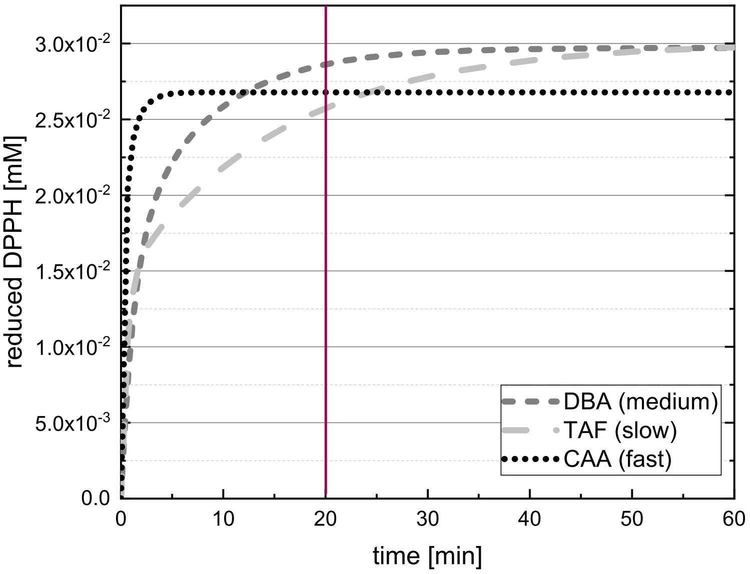


Figure 3. Representative fit curves showing the quantity of reduced DPPH radical ($\Delta[\text{DPPH}\cdot]_t = [\text{DPPH}\cdot]_0 - [\text{DPPH}\cdot]_t$) in reactions with DBA, CAA and TAF as a function of time, demonstrating different kinetic behaviors.

If the value of DBA or TAF is measured after 20 min in the absence of kinetic data, the true value can therefore be underestimated, and in some cases a different kinetic order for the antioxidant effect might be predicted. Accordingly, we checked whether the extrapolated final values differed significantly when the measured values at 13, 17 and 20 min were used to adjust Equation (12). If there was a significant difference between the values predicted at these time points, the assay was repeated with longer measurement durations. Figure 4 shows the value reported for EPC after 14 h.

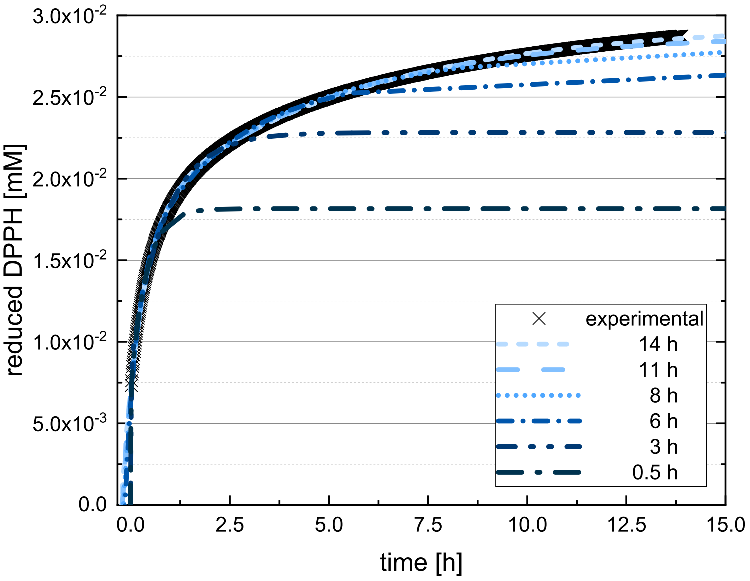


Figure 4. Representative measurement curve of the quantity of reduced DPPH radical in reactions with EPC as a function of time. Equation (12) is adapted for different time periods.

Here, there was no significant difference between the calculated final values after adapting Equation (12) using the values measured at 11 and 14 h so the measurement duration was considered sufficient. The substances that required longer measurement durations during the DPPH assay and ABTS assay are discussed in more detail below. In the following, we report the results for the subgroups using boxplots for the DPPH (Figure 5) and ABTS (Figure 6) assays and the results of all used phenolic compounds in both assays (Figure 7).

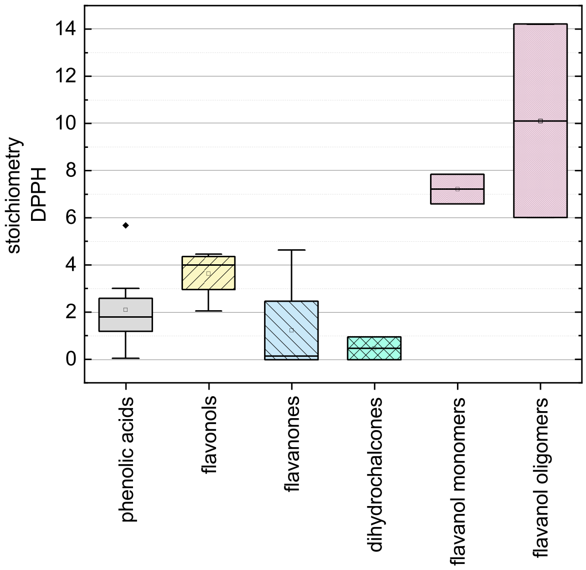


Figure 5. Boxplot of the mean stoichiometry values for subgroups of the phenolic compounds tested in the DPPH assay. Error bars represent range within 1.5 × IQR.

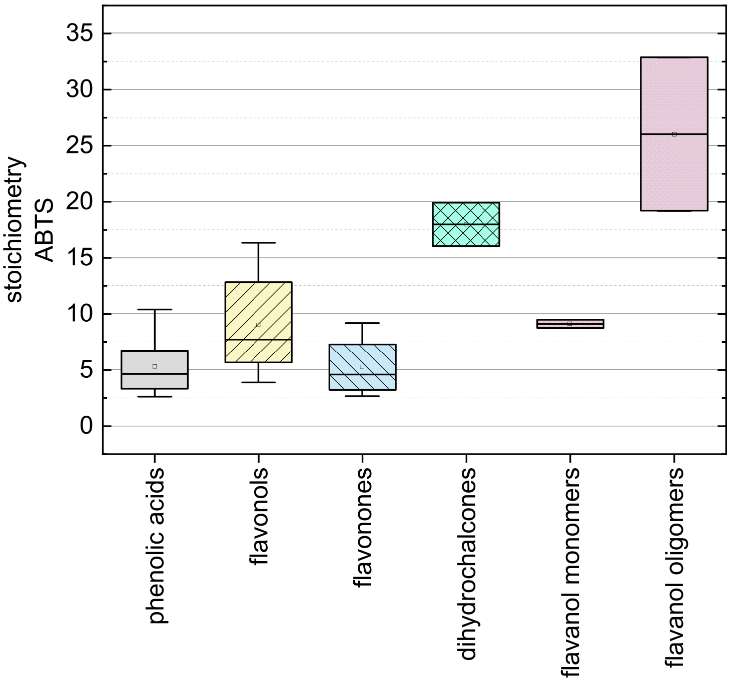


Figure 6. Boxplot of the mean stoichiometry values for subgroups of the phenolic compounds tested in the ABTS assay. Error bars represent range within 1.5 × IQR.

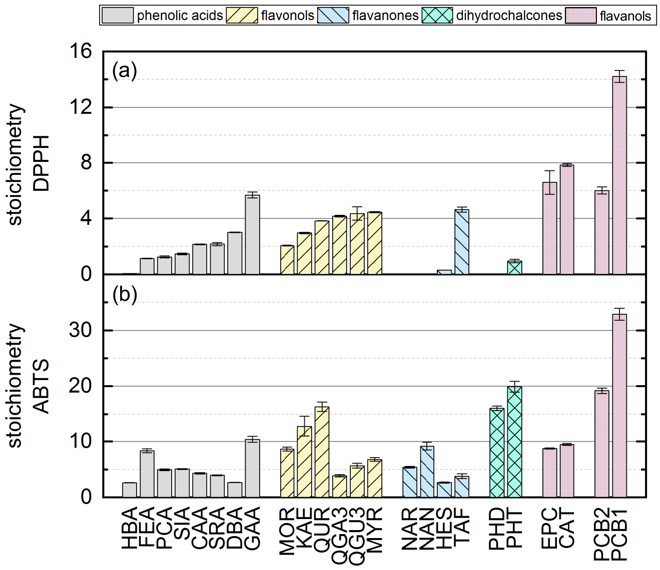


Figure 7. The antioxidant activity of all standard references in the (**a**) DPPH and (**b**) ABTS assays. Data are mean values of three measurements and standard errors.

3.1. Antioxidant Activity Determined Using the DPPH Test

The antioxidant activities based on the DPPH assay are shown as stoichiometry boxplots in Figure 5. The sequence of decreasing mean values was: flavanol oligomers > flavanol monomers > flavonols > phenolic acids > flavanones > dihydrochalcones, which is largely consistent with previous reports with the exception of the dihydrochalcones [34]. The order of the flavonoid subgroups mainly depended on the number and position of OH groups, which is why the flavanol oligomers (PCB1 and PCB2) consisting of two flavanol monomers (EPC and CAT) achieved a higher mean value [34]. In addition, the Bors criteria appeared to play a decisive role [7]. Accordingly, the flavanols display high antioxidant values because they fulfill Bors criterion 1, which seems to be the most important of the three [35]. Similarly, the flavonols fulfill Bors criterion 2 and most of them fulfill Bors criterion 3 and also carry a catechin or pyrogallol group on the B-ring. Phenolic acids are difficult to compare with flavonoids because these groups are structurally distinct, but we observed a wide range of antioxidant values in part reflecting the high values assigned to hydroxybenzoic acids but the lower values assigned to hydroxycinnamic acids. The low mean value of the flavanones reflects the low number of OH groups and the two compounds (NAR and NAN) that did not react with the DPPH radical. The dihydrochalcones had the lowest values overall, reflecting the low number of OH groups and the open C-ring of PHT. Furthermore, PHD did not react with the DPPH radical in our assay. Because most of the compounds fulfill Bors criterion 3, it is not possible to make a definitive statement concerning its influence on the SAR.

The individual results of all reference standards in the DPPH assay are shown in Figure 7a and depend mainly on Bors criterion 1. All substances with a catechol or pyrogallol group on the B-ring showed high antioxidant values. In contrast to Bors' statement that substances meeting all three criteria are the most potent antioxidants [7], MYR and QUR achieved only medium-high activity and the most active compounds in our DPPH assay fulfilled only Bors criterion 1. HBA, FEA, PCA, SIA and SRA each possess only one OH group, and these showed low antioxidant values. An OH group in the *para* position confers more activity than one in the *meta* position, which is why HES also showed low activity [23,34,36–41]. The references NAR, NAN and PHD did not react with the DPPH radical, perhaps due to steric hindrance. The stoichiometry and kinetic behavior of all reference standards are summarized in Table 2.

Table 2. Results of the DPPH assay presented as stoichiometry values after 5 and 30 min and stationary final values with literature comparisons and kinetic behaviors. Data are mean values of three measurements and standard errors. Sample names and structures are shown in Table 1.

Group	Sample Code	Stoichiometry (5 min)	Stoichiometry (30 min)	Stoichiometry (Steady Sate)	Kinetic Behavior	Stoichiometry (Literature)	Sources
phenolic acids	CAA	2.20 ± 0.06	2.24 ± 0.04	2.15 ± 0.03	fast	1.7–4.54	[18,23,27,42]
	DBA	2.51 ± 0.04	2.93 ± 0.11	3.01 ± 0.02	medium	2.1–2.8	[27,43,44]
	FEA	0.78 ± 0.02	1.11 ± 0.02	1.14 ± 0.01	medium	1.1–1.39 ^{d}	[23,27,29,42]
	GAA	5.13 ± 0.26	5.71 ± 0.23	5.68 ± 0.21	fast	5.6–7.14 ^{d}	[23,27,29]
	HBA	0.07 ± 0.00	0.06 ± 0.01	0.06 ± 0.01	fast	0	[35]
	PCA	0.14 ± 0.01	0.27 ± 0.03	1.25 ± 0.07	slow	0.9	[18]
	SIA	1.26 ± 0.04	1.31 ± 0.03	1.46 ± 0.06	medium	1.2 ^{d}	[18]
	SRA	1.57 ± 0.10	2.12 ± 0.10	2.17 ± 0.10	medium	2.7	[27]
flavonols	KAE	2.96 ± 0.05	2.91 ± 0.05	2.96 ± 0.04	fast	1.8	[27]
	MOR	1.95 ± 0.02	2.05 ± 0.03	2.06 ± 0.03	medium	1.8	[27]
	MYR	3.26 ± 0.21	4.38 ± 0.03	4.46 ± 0.04	medium	7.6	[27]
	QGA3	2.33 ± 0.10	4.01 ± 0.09	4.17 ± 0.05	medium	-	
	QUR	2.27 ± 0.13	3.81 ± 0.02	3.83 ± 0.02	medium	4.86–5.2	[27,28]
	QGU3	3.23 ± 0.08	4.43 ± 0.27	4.36 ± 0.47	medium	3.78	[28]
flavonones	HES	0.12 ± 0.01	0.27 ± 0.01	0.30 ± 0.10	slow	0.9	[45]
	NAN	0	0	0	-	0	[35]
	NAR	0	0	0	-	-	
	TAF	2.89 ± 0.06	4.15 ± 0.09	4.63 ± 0.19	slow	4.18 ^{d}	[46]
dihydrochalcones	PHD	0	0	0	-	-	
	PHT	0.16 ± 0.01	0.65 ± 0.03	0.96 ± 0.13	slow	-	
flavanols	CAT	2.39 ± 0.09	3.58 ± 0.05	7.84 ± 0.10	slow	3.72–4.5	[27,28]
	EPC	1.84 ± 0.01	2.51 ± 0.16	6.58 ± 0.84	slow	3.96–6.6	[27,28]
	PCB1	8.83 ± 0.13	12.29 ± 0.25	14.21 ± 0.42	slow	7.6	[27]
	PCB2	4.19 ± 0.36	5.81 ± 0.13	6.01 ± 0.26	medium	7.4	[27]

^{d} converted from EC$_{50}$.

For the **phenolic acids**, the ranking of antioxidant activity was GAA $\gg$ DBA $>$ SRA $\approx$ CAA $>$ SIA $>$ PCA $>$ FEA $\gg$ HBA in broad agreement with the literature [34,41,47,48]. In the case of PCA, the extrapolated final values differed significantly after adjusting the curve to 1000 and 1200 s using Equation (12), so we extended the duration of measurement. The results depended mainly on the number of OH groups on the aromatic ring, which is why GAA with three groups was more active than DBA and CAA, which both possess a catechol group. Substances with only one OH group had the weakest antioxidant activity (SIA, PCA, FEA and HBA). GAA showed the highest values due to its pyrogallol-like structure and ability to release hydrogen atoms, which is particularly important for hydrogen transfer [34,40]. As stated above, OH groups at the *para* position confer high antioxidant activity [23,34,36–41]. We found that hydroxybenzoic acids (GAA, DBA and SRA) were more potent than hydroxycinnamic acids (CAA, SIA, FEA and PCA), also in agreement with the literature [34,49,50]. Hydroxycinnamic acid could play a major role in the stabilization of the radical by resonance and also has a large hydrogen release capacity, leading to the stabilization of the resulting radical [34]. The order for the hydroxycinnamic acids we tested was SRA $>$ SIA $>$ FEA $>$ PCA, suggesting that the methoxy group also influences stoichiometry in the DPPH assay, as previously reported [34,40]. The methoxy group is an electron donor, reducing the bond dissociation energy and therefore promoting electron transfer [13].

GAA, CAA and HBA were the phenolic acids with the fastest kinetics, partly in agreement with the literature [42,51,52]. The presence of a catechol, pyrogallol or single OH group appeared to have little effect on reaction kinetics. Phenolic acids in the medium group (SRA, SIA, DBA and FEA) did not react completely within 30 min, which may lead to an underestimation of their antioxidant activity. FEA was shown to have medium kinetics in a previous study [29]. PCA was the only phenolic acid with a slow kinetics in our assay.

For the **flavonols**, the ranking of antioxidant activity was MYR > QGU3 > QGA3 > QUR > KAE > MOR, when the compounds were dissolved in ethanol. Another study reported a similar order to that observed in our experiments (MYR > QUR > MOR = KAE) [27]. Because flavonols fulfill Bors criterion 2 and in many cases also criterion 3, we do not discuss them further here. The highest activity was observed for substances with an additional catechol group on the B-ring, which also corresponds to the Bors criteria. MYR has catechol and OH groups, thus explaining its slightly higher value than QUR (with a lone catechol group) but indicating that the additional OH group has only a small influence. The presence of a sugar residue likewise has only a small influence, explaining why the two glycosylated quercetins showed similar values to the quercetin aglycone. In methanol, the sugar residues were found to slightly inhibit antioxidant activity [35]. KAE and MOR have no catechol group and therefore showed the lowest values in our assay. All substances showed medium kinetics except KAE, which was assigned to the fast kinetics group. In contrast to the phenolic acids, the presence of catechol or pyrogallol groups did appear to affect the reaction velocity, explaining why the quercetin derivatives and MYR behaved in the same manner. All substances assigned to the medium kinetics category featured two OH groups on the B-ring.

For the **flavanols**, the ranking of antioxidant activity was PCB1 > CAT > EPC ≈ PCB2. EPC and CAT are structural isomers and were measured over a longer duration because data from the 30-min assay were insufficient. EPC and CAT produced similar yet significantly different activities. PCB1 and PCB2 are also structural isomers, but PCB2 showed a much lower antioxidant activity probably due to steric hindrance. PCB2 was assigned to the medium kinetics category, whereas PCB1, EPC and CAT were assigned to the slow kinetics category.

For the **flavanones**, only two of the four standard references reacted with the DPPH radicals, with TAF showing much greater activity than HES. NAR and NAN are structurally similar, but NAR carries an additional sugar residue at position 7 on the A-ring, which could prevent interaction with DPPH due to steric hindrance. The minimal activity of HES requires further investigation. TAF fulfills Bors criteria 1 and 3, thus achieving a higher value than HES. Furthermore, HES features only one OH group (in the *meta* position of the B-ring) in contrast to the catechol group of TAF. Both TAF and HES showed slow kinetic behavior.

Finally, we tested two **dihydrochalcones** (PHD and PHT) only the latter of which reacted with DPPH. PHD and PHT are structurally similar, differing only in the presence of a sugar residue at position 6′ on the A-ring of PHD, which as stated above for NAR could prevent interaction with the DPPH radical due to steric hindrance. PHT showed a low reaction stoichiometry and slow kinetic behavior. PHD has been reported to react moderately or slowly with DPPH, but these experiments involved different solvents [42,53].

In summary, our DPPH assay results partly agree with the literature, with discrepancies likely to reflect the different solvents used and other differences in the measurement and/or evaluation methods. Our results showed no clear correlation between the structure of the polyphenolic molecules and the reaction rate. The intermediate product AO· cannot be distinguished from the final product by spectrophotometry, hence the speeds of the two reactions and the reaction rates $\widetilde{k}_1$ and $\widetilde{k}_2$ cannot be determined individually. The assignment of reaction speeds [23] therefore indicates an average value for both reactions. To achieve a high value in the assay, the three Bors criteria and the number of OH groups on the molecule appear decisive. Furthermore, the presence of an OH group in the *para* position of the B-ring is important and the presence of a sugar residue has a positive effect, regardless of the type of sugar [23,34,36–41].

3.2. Antioxidant Activity Determined Using the ABTS Test

The antioxidant activities based on the ABTS assay are shown as stoichiometry boxplots in Figure 6. The sequence of decreasing mean values was: flavanol oligomers

> dihydrochalcones > flavanol monomers > flavonols > phenolic acids > flavanones, which again is largely consistent with the literature with the exception of the dihydrochalcones [34]. In general, the number of OH groups seemed to determine the order. The high values of dihydrochalcones may reflect the lower steric hindrance of their relatively open structures. When comparing all 24 substances regardless of their subgroup, there were no conspicuous structural features that explained the ranking [37]. The stoichiometry and kinetic behavior of all the reference standards are shown in Table 3. However, ABTS results from literature are not shown because they are usually provided as TEAC values, which correlate poorly with the reaction kinetics [54]. The individual results of all reference standards in the ABTS assay are shown in Figure 7b.

For the **phenolic acids**, the ranking of antioxidant activity was GAA ≫ FEA > SIA ≈ PCA > CAA > SRA > DBA ≈ HBA. GAA achieved the highest value due to the pyrogallol group on the aromatic ring. However, the presence of a single OH or catechol group appeared to be less important, explaining the similar values of HBA vs DBA and CAA vs PCA, all lower than GAA. Furthermore, the hydroxybenzoic acids appeared more active than the hydroxycinnamic acids, the exception being the benzoic acid GAA, which achieved the highest value. The presence of a methoxy group also affected the activity, explaining why SRA, SIA and FEA achieved higher values than HBA and PCA. FEA, which has two methoxy groups, achieved a higher value than SIA with only one. CAA, FEA, GAA and SIA were assigned to the medium kinetics category, whereas all the others showed fast kinetics. There was no clear correlation between the reaction velocity and chemical structure, and the type of acid group appeared to have no influence on the velocity of the reaction.

Table 3. Results of the ABTS assay presented as stoichiometry values after 5 and 30 min and stationary final values and kinetic behaviors. Data are mean values of three measurements and standard errors. Sample names and structures are shown in Table 1.

Group	Sample Code	Stoichiometry (5 min)	Stoichiometry (30 min)	Stoichiometry (Steady Sate)	Kinetic Behavior
phenolic acids	CAA	3.93 ± 0.14	4.35 ± 0.10	4.31 ± 0.13	medium
	DBA	2.60 ± 0.06	2.61 ± 0.06	2.66 ± 0.04	fast
	FEA	6.14 ± 0.25	8.46 ± 0.41	8.34 ± 0.37	medium
	GAA	8.47 ± 0.35	10.77 ± 0.50	10.38 ± 0.54	medium
	HBA	2.60 ± 0.02	2.59 ± 0.02	2.61 ± 0.02	fast
	PCA	4.58 ± 0.38	5.17 ± 0.23	4.95 ± 0.16	fast
	SIA	4.21 ± 0.06	5.04 ± 0.08	5.07 ± 0.08	medium
	SRA	3.98 ± 0.06	4.21 ± 0.05	3.96 ± 0.08	fast
flavonols	KAE	3.89 ± 0.46	5.33 ± 0.74	12.81 ± 1.82	slow
	MOR	8.64 ± 0.36	8.96 ± 0.50	7.45 ± 0.34	medium
	MYR	6.09 ± 0.16	6.86 ± 0.22	6.78 ± 0.18	medium
	QGA3	2.79 ± 0.09	3.69 ± 0.18	3.88 ± 0.20	medium
	QGU3	4.29 ± 0.19	5.84 ± 0.39	5.64 ± 0.43	medium
	QUR	9.67 ± 0.82	11.38 ± 0.28	12.42 ± 0.64	slow
flavonones	HES	2.93 ± 0.16	3.11 ± 0.11	2.66 ± 0.09	fast
	NAN	2.68 ± 0.21	4.63 ± 0.44	9.17 ± 0.69	slow
	NAR	0.69 ± 0.22	2.28 ± 0.34	5.39 ± 0.14	slow
	TAF	3.14 ± 0.12	3.76 ± 0.39	3.76 ± 0.43	fast
dihydrochalcones	PHD	5.20 ± 0.07	9.08 ± 0.06	16.05 ± 0.40	slow
	PHT	6.50 ± 0.29	12.56 ± 0.50	19.90 ± 0.96	slow
flavanols	CAT	9.30 ± 0.18	10.00 ± 0.26	9.47 ± 0.18	medium
	EPC	8.16 ± 0.05	8.78 ± 0.14	8.74 ± 0.12	medium
	PCB1	26.57 ± 0.41	31.48 ± 0.52	32.87 ± 1.06	medium
	PCB2	15.22 ± 0.23	18.61 ± 0.45	19.19 ± 0.50	medium

For the **flavonols**, the ranking of antioxidant activity was QUR > KAE > MOR > MYR > QGU3 > QGA3. As discussed for the phenolic acids, the activity of the flavonols does not appear to depend on the presence of the catechol group, which is why QUR and

KAE reached similar values. Additional OH groups had a negative impact, hence MYR achieved a much lower value than QUR, possibly caused by steric hindrance. Furthermore, the presence of any sugar residue also caused a negative impact. A second OH group on the B-ring only influenced the activity if it formed a catechol group, which is why MOR did not show a higher value than KAE. The flavonol standard references showed medium kinetics, except QUR and KAE, which were assigned to the slow kinetics category. For these two substances, it was necessary to repeat the measurement with an extended duration, although it is unclear which chemical groups were responsible for the effect. There may be steric hindrance in some molecules, for example due to the presence of a sugar residue or a pyrogallol group.

For the **flavanols**, the ranking of antioxidant activity was PCB1 $>$ PCB2 $>$ EPC $\approx$ CAT. As stated above, EPC and CAT are structural isomers and so are PCB1 and PCB2. PCB2 consists of two EPC molecules and thus has twice the number of catechol and OH groups, explaining its higher activity than EPC/CAT. PCB1 achieved the highest value overall, suggesting that PCB2 again suffers from a steric hindrance effect. All standard references showed medium kinetic behavior, which was expected due to the structural similarity of the molecules.

For the **flavanones**, the ranking of antioxidant activity was NAN $>$ NAR $>$ TAF $>$ HES. The order appeared to depend mainly on the position of the OH group in the B-ring. HES, with its *meta* OH group, showed the lowest activity, whereas the more active TAF, NAR and NAN all have an OH group in the *para* position [23,34,36–41]. The presence of a sugar residue appeared to inhibit antioxidant activity, hence the lower value of NAR compared to NAN. TAF achieved a higher value than NAN because it fulfills the second and third Bors criteria. NAR and NAN were assigned to the slow kinetics category requiring a longer duration of measurement, whereas TAF and HES showed fast kinetics.

Finally, we found that both **dihydrochalcones** (PHD and PHT) were active in the ABTS assay, although PHT was more active than PHD. The only difference between these compounds is the presence of a sugar residue on the A-ring of PHD, which appears to exert a negative influence on antioxidant activity. Both PHD and PHT showed slow kinetic behavior and were analyzed by extending the measurement duration.

In summary, it was not possible to compare the sequences of all substances because different trends were observed within each group, hence no SAR and no clear correlation between molecular structure and reaction rate in the assay could be established. The precise structural properties that are important in the ABTS assay could not be determined, and the Bors criteria seem to play a minor role. The kinetic behavior of the reaction reflects the complexity of the reactant, with large polyphenolic molecules reacting more slowly than simpler ones because the former must reorient before reaction with the ABTS radical. However, substances that show fast kinetics can also have low antioxidant capacities, and substances that show a high stoichiometry with the ABTS radical do not necessarily achieve high reactivity [54].

3.3. Comparison of the DPPH and ABTS Assays

When comparing the boxplots (Figures 5 and 6), the mean values of the polyphenol subgroups, with the exception of the dihydrochalcones, revealed similar trends in both assays. The subgroup of the dihydrochalcones showed radically different mean values, reflecting the fact that one of the two tested compounds did not react with the DPPH radical.

We observed no clear correlation when comparing the results of the individual substances in the two assays, suggesting that antioxidant activity is dependent on multiple criteria (Figure 7).

Results in the DPPH assay appeared to depend mainly on the number of OH groups and Bors criteria 1 and 3, but further investigation is required because most of the standard reference compounds we used conformed at least to Bors criterion 3. In contrast, there was no clear relationship between the results in the ABTS assay and the number of OH groups, and the Bors criteria were much less important.

For the **phenolic acids**, the hydroxycinnamic acids achieved higher values than the hydroxybenzoic acids in the ABTS assay, whereas this was not the case in the DPPH assay. Furthermore, compounds with an additional methoxy group showed higher activities in both assays. However, compounds with both OH and methoxy groups achieved higher values than those with a catechol group in the ABTS assay but not in the DPPH assay. For the DPPH assay, activity was mainly dependent on the number of OH groups and the presence of catechol or pyrogallol groups.

For the **flavonols**, the first Bors criterion was important in both assays. An additional OH group on the B-ring increased the activity in the DPPH assay but not the ABTS assay. The presence of a sugar residue conferred a slightly negative effect in the ABTS assay but had no significant impact on the DPPH assay. The presence of an OH group at position 2 did not affect the results of either assay.

For the **flavanones**, the presence of an OH group at position 3 in the C-ring had a major effect on the results of the DPPH assay, and the presence of a sugar residue tended to abolish the reaction. The *para* position of an OH group in the B-ring appeared to play an important role in both assays.

Among the four **flavanols** we tested, PCB1 achieved high values in both assays, perhaps due to the high number of catechol and OH groups. In contrast, PCB2 showed high activity only in the ABTS assay, suggesting steric hindrance may inhibit its activity in the DPPH assay. CAT and EPC are structural isomers that do not seem prone to steric hindrance, thus the differences between these compounds were small in both assays.

Finally, although we tested only two **dihydrochalcones**, making it difficult to draw general conclusions for this group of molecules, it appears that the sugar residue on PHD had a negative impact on activity, and this aspect could be explored by testing more diverse compounds.

4. Conclusions

The mean values of most of the polyphenol subgroups revealed similar trends in both assays. The dihydrochalcones were the only compounds to show radically different mean values, reflecting the fact that we tested only two compounds and one of them did not react with the DPPH radical. Therefore, dihydrochalcone-rich extracts should not be measured using the DPPH assay because this underestimates the antioxidant activity. The same caveat applies to the flavanones. If these substances are divided into subgroups, the assays reported different results despite the identical measurement and evaluation methods. In general, the results of the DPPH assay correlated mainly with the Bors criteria, whereas the SAR was not clear in the ABTS assay. This may be due the type of solvent, which was not possible to be the same in the two assays. Furthermore, the DPPH and ABTS model radicals are structurally distinct. In terms of kinetic behavior, we observed no clear correlation between structure and reaction velocity. However, to ensure that endpoint values are reported, the duration of measurement should be at least 30 min and Equation (12) should be used to calculate the final values, thus avoiding measurement times of several hours. This also ensures that the measurement duration can be extended if necessary. Additionally, the potential synergistic effects of pure substances should be taken into account when measuring the reaction kinetics of extracts containing mixtures of substances that react at different speeds. In order to use these assays as rapid tests for specific applications, they must be matched to the antioxidant effect required in the application medium (e.g., food matrix or body tissue). Other relevant parameters such as pH, solvent, and the content of carbohydrate and protein should also be considered, because these may affect the reported activity of natural extracts.

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Abbreviations

The following abbreviations are used in this manuscript:

ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
AOH	antioxidant
ARP	antiradical power
CAA	caffeic acid
CAT	(+)-catechin
DBA	3,4-dihydroxybenzoic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	half maximal effective concentration
EPC	(−)-epicatechin
FEA	ferulic acid
GAA	gallic acid
HAT	hydrogen atom transfer
HBA	4-hydroxybenzoic acid
HES	hesperetin
KAE	kaempferol
MOR	morin
MYR	myricetin
NAN	naringenin
NAR	narirutin
ORAC	oxygen radical absorbance capacity
PCA	p-coumaric acid
PCB1	proanthocyanidin B1
PCB2	proanthocyanidin B2
PHD	phloridzin
PHT	phloretin
QGA3	quercetin-3-D-galactosides
QGU3	quercetin-3-D-glucosides
QUR	quercetin
SAR	structure-activity relationship
SET	single electron transfer
SET-PT	single electron transfer followed by a proton transfer
SIA	sinapic acid
SPLET	sequential proton loss electron transfer
SRA	siringic acid
TAF	taxifolin
TEAC	trolox equivalent antioxidant capacity

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4.2 How does the phenol structure influence the results of the Folin-Ciocalteu assay? [2]

Summary

In addition to the ABTS and DPPH assay, there are other SET-based *in vitro* assays for determining antioxidant activity, such as the FC assay. This assay was originally used to measure the total polyphenol content of extracts, and although it is already known from the literature that the antioxidant effect is measured but not the polyphenol content, it is often still used for the determination of the total polyphenol content. Therefore, it is not clear from the literature for which type of result can be obtained from this assay. Thus, since there are different opinions in the literature about the suitability of the assay, little is also known about the structure-activity relationships as well as comparability with other SET-based assays. For this reason, again 24 different phenolic components were measured in the form of reference standards (phenolic acids, flavonols, flavanols, dihydrochalones, and flavanones) to identify the structurally crucial properties for a high value in the assay.

It was found that the FC is also suitable for determining the antioxidant effect of phenol-rich samples and did not measure the total polyphenol content. Furthermore, the FC assay is particularly influenced by the number of hydroxyl groups in a molecule, as well as the Bors criteria. Based on these results, it was possible to define five new classes based on a combination of the influencing factors, with class 1 achieving the lowest and class 5 the highest value in the FC assay.

- class 1: 2 OH groups and none of the Bors criteria
- class 2: 3 to 4 OH groups and none of the Bors criteria
- class 3: one of the Bors criteria
- class 4: two of the Bors criteria
- class 5: all of the Bors criteria

This new classification made it possible to predict the effect of a substance based on its chemical structure.

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[2] Platzer M., Kiese S., Herfellner T., Schweiggert-Weisz U., Eisner P., How does the phenol structure influence the results of the Folin-ciocalteu assay?, *Antioxidants* **10**, 811 (2021)

Furthermore, no correlation of FC results with those of other SET-based assays (ABTS and DPPH assay) was found in this study, although the assays were partly influenced by the same structural properties. While the results in the FC and the DPPH assays were strongly influenced by the number of hydroxyl groups as well as the Bors criteria, these factors were not crucial for the ABTS assay. Despite some similarities between the DPPH and FC assay, different results were also obtained and no clear trend could was shown. In contrast to the DPPH assay, the FC assay was also suitable for the measurement of dihydrochalcones and flavanones and should therefore preferably be used for extracts with unknown composition.

Author contributions:

- Melanie Platzer: conceptualization, methodology, data curation, writing-original draft preparation and visualization
- Sandra Kiese: conceptualization, methodology, writing-review and editing, visualization and supervision
- Thomas Herfellner: writing-review and editing, supervision and funding acquisition
- Ute Schweiggert-Weisz: writing-review and editing and supervision
- Peter Eisner: validation and writing-review and editing and supervision

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Article

How Does the Phenol Structure Influence the Results of the Folin-Ciocalteu Assay?

Melanie Platzer [1,2,*], Sandra Kiese [2], Thomas Herfellner [2], Ute Schweiggert-Weisz [2,3] and Peter Eisner [1,2,4]

[1] ZIEL-Institute for Food & Health, TUM School of Life Sciences Weihenstephan, Technical University of Munich, Weihenstephaner Berg 1, 85354 Freising, Germany; peter.eisner@ivv.fraunhofer.de
[2] Fraunhofer Institute for Process Engineering and Packaging IVV, Giggenhauser Str. 35, 85354 Freising, Germany; sandra.kiese@ivv.fraunhofer.de (S.K.); thomas.herfellner@ivv.fraunhofer.de (T.H.); ute.weisz@ivv.fraunhofer.de (U.S.-W.)
[3] Chair of Food Science, Institute for Nutritional and Food Sciences, University of Bonn, Meckenheimer Allee 166a, 53113 Bonn, Germany
[4] Faculty of Technology and Engineering, Steinbeis-Hochschule, George-Bähr-Str. 8, 01069 Dresden, Germany
* Correspondence: melanie.platzer@ivv.fraunhofer.de

Abstract: Plants produce a diverse array of secondary metabolites that are generally nonessential but facilitate ecological interactions. Fruits, vegetables, seeds and nuts can accumulate bioactive secondary metabolites with health-promoting properties, including the potent antioxidant activities of phenolic compounds. Several in vitro assays have been developed to measure the polyphenol content and antioxidant activity of plant extracts, e.g., the simple and highly popular Folin-Ciocalteu (FC) assay. However, the literature contains a number of different descriptions of the assay and it is unclear whether the assay measures the polyphenol content or reducing capacity of the sample. To determine the influence of phenolic structures on the outcome of the FC assay, we tested phenols representing different subgroups (phenolic acids, flavonols, flavanols, dihydrochalcones and flavanones). We observed different results for each reference substance and subgroup. Accordingly, we concluded that the FC assay does not measure the polyphenol content of a sample but determines its reducing capacity instead. Assigning the substances to five structural classes showed that the FC results depend on the number of fulfilled Bors criteria. If a molecule fulfills none of the Bors criteria, the FC results depend on the number of OH groups. We did not find a correlation with other single electron transfer assays (e.g., ABTS and DPPH assays). Furthermore, the FC assay was compatible with all five subgroups and should be preferred over the DPPH assay, which is specific for extracts rich in dihydrochalcones or flavanones.

Keywords: reducing capacity; antioxidant effect; flavonoids; phenolic acids; structure-activity relationship

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1. Introduction

Plants produce tens of thousands of secondary (specialized) metabolites that are not required for basic cellular functions or normal growth and development, but fulfill various ecological roles including protection from herbivores, pests, pathogens and UV radiation [1–3]. Secondary metabolites that accumulate in the edible parts of plants are known for their health-promoting and disease-preventing properties in humans, attracting interest from the pharmaceutical, food and nutritional supplement industries. Many secondary metabolites are potent antioxidants, with the ability to scavenge reactive oxygen species and other free radicals. These compounds can protect foods from oxidation and are widely used as additives to improve food quality and storage stability [1,4–8].

Phenolic compounds are among the most important antioxidants found in plants. This diverse group includes low-molecular-weight compounds with a single aromatic ring, such as phenolic acids, as well as large and complex polyphenolic molecules, such as flavonoids [9,10]. Two important structural subgroups of the phenolic acids are the hydroxybenzoic acids and the hydroxycinnamic acids, which are found in almost all plant-based foods and are thought to promote protein synthesis and nutrient absorption. The most common phenolic acids are ferulic acid, *p*-coumaric acid, gallic acid and caffeic acid [11,12]. Flavonoids typically consist of two or more aromatic rings, each carrying at least one aromatic hydroxyl group, with the rings connected by a carbon bridge [13]. They are divided into four subgroups (flavonols, flavanols, dihydrochalcones and flavanones) based on the connection between the B- and C-rings as well as the oxidation state and the functional groups on the C-ring [14]. The phenolic compounds considered in this study are summarized in Table 1 (slightly modified from Table 1 in Platzer et al. [15] with permission).

The antioxidant activity of these compounds depends on their overall structure as well as the position and type of side groups, which is known as the structure-activity relationship (SAR) [16]. Three criteria have been proposed to explain the antioxidant behavior of phenolic compounds, and these are known as Bors criteria [17]. The first is the presence of a catechol group on the B-ring (Bors 1), which increases the stability of the resulting antioxidant radical. The second is the presence of a 2,3 double bond combined with a 4-oxo group on the C-ring (Bors 2), which facilitates electron delocalization. The third is the presence of OH groups at positions 3 and 5 in combination with a 4-oxo group, which enables electron delocalization via hydrogen bonds (Bors 3). The three Bors criteria are summarized in Figure 1.

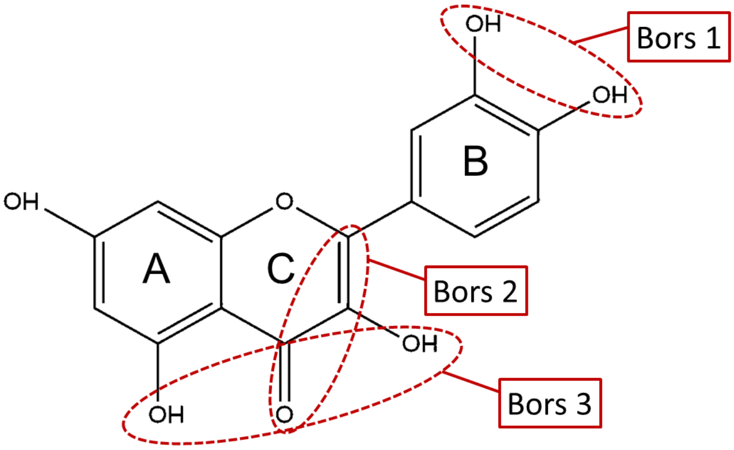
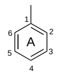
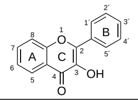
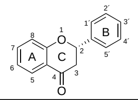
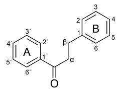
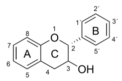


Figure 1. Structure activity relationship based on Bors criteria. Bors 1—catechol group on the B-ring; Bors 2—2,3 double bond and 4-oxo group on the C-ring; Bors 3—OH groups at position 3 and 5 OH group on the A- and C-rings and 4-oxo group on the C-ring.

The antioxidant activity of phenolic compounds is often determined using a rapid assay. Popular tests include the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC) and Folin-Ciocalteu (FC) photometric assays [18–21]. These are based on either single electron transfer (SET) or hydrogen atom transfer (HAT) reaction mechanisms [22,23]. The ABTS, DPPH and FC assays follow a SET mechanism and opinions vary as to whether there is a correlation between the results of these assays [21,23–26]. Common trends and differences between the ABTS and DPPH assays have been reported previously [15].

The FC assay was originally developed for the detection and quantification of tyrosine [27] and was later modified to measure the polyphenol content of red wine [28].

Table 1. The phenolic compounds analyzed in this study (phenolic acids and subgroups of flavonoids) along with reference standards, character codes, and side groups (reproduced and slightly modified from Table 1 in Platzer et al. [15] with permission).

Subgroup	Reference Standard	Sample Code	Class	Side Group						
phenolic acids				**1**	**3**	**4**	**5**			
	caffeic acid	CAA	-	$(CH_2)_2COOH$	OH	OH	H			
	3,4-dihydroxybenzoic acid	DBA	-	COOH	OH	OH	H			
	ferulic acid	FEA	-	$(CH_2)_2COOH$	OH	OCH_3	H			
	gallic acid	GAA	-	COOH	OH	OH	OH			
	4-hydroxybencoic acid	HBA	-	COOH	H	OH	H			
	p-coumaric acid	PCA	-	$(CH_2)_2COOH$	H	OH	H			
	sinapic acid	SIA	-	$(CH_2)_2COOH$	OCH_3	OH	OCH_3			
	siringic acid	SRA	-	COOH	OCH_3	OH	OCH_3			
flavonols				**2′**	**3′**	**4′**	**5′**	**3**	**5**	**7**
	isorhamnetin	IRT	4	H	OH	OH	H	OH	OH	OH
	kaempferol	KAE	4	H	H	OH	H	OH	OH	OH
	morin	MOR	4	OH	H	OH	H	OH	OH	OH
	quercetin-3-D-galactoside	QGA3	4	H	OH	OH	H	Glc	OH	OH
	quercetin-3-D-glucoside	QGU3	4	H	OH	OH	H	Gal	OH	OH
	quercetin-7-D-glucoside	QGU7	5	H	OH	OH	H	OH	OH	Glc
	quercetin	QUR	5	H	OH	OH	H	OH	OH	OH
flavanones				**3′**	**4′**	**3**	**5**	**7**		
	hesperetin	HES	2	OH	OCH_3	H	OH	OH		
	narirutin	NAR	1	H	OH	H	OH	2 Glc		
	naringin	NAG	1	H	OH	H	OH	Rham, Glc		
	naringenin	NAN	2	H	OH	H	OH	OH		
	taxifolin	TAF	4	OH	OH	◄OH	OH	OH		
dihydrochalcones				**5**	**7**	**9**	**4′**			
	phloridzin	PHD	2	OH	OH	OH	Glc			
	phloretin	PHT	2	OH	OH	OH	OH			
flavanols				**3′**	**4′**	**3**	**4**	**5**	**7**	
	(+)-catechin	CAT	3	OH	OH	⋯OH	H	OH	OH	
	(-)-epicatechin	EPC	3	OH	OH	◄OH	H	OH	OH	

The assay quickly gained popularity due to its simplicity and reproducibility. When the FC reagent is exposed to an antioxidant, it is reduced to a blue complex that can be quantified by spectrophotometry. The exact composition of the reagent is not published, but it probably consists of a mixture of heteropolyacid, phosphomolybdenum and phosphotungstic acid. The color change is thought to reflect the reduction of molybdenum, as shown in Equations (1) and (2) [23,28].

$$\mathrm{Mo}^{+6}\,(\mathrm{yellow}) + \mathrm{e}^{-1} \longrightarrow \mathrm{Mo}^{+5}\,(\mathrm{blue}) \tag{1}$$

$$\mathrm{Mo}^{+5} + \mathrm{e}^{-1} \longrightarrow \mathrm{Mo}^{+4}\,(\mathrm{blue}) \tag{2}$$

In the literature, the FC assay is often described as a means to determine the total polyphenol content and is typically used for the analysis of plant extracts and juices [27,29,30]. The SAR is rarely mentioned because extracts and juices are complex mixtures of many phenolic and nonphenolic components, which makes the effect of particular structures impossible to isolate. Some authors have suggested that FC assay results depend on the number and position of OH groups, particularly those on the B-ring [31–33]. The OH group at position 3 on the A-ring also appears to play an important role [31]. Furthermore, OH groups in the *para* or *ortho* positions appear to confer stronger reducing capacity than those in the *meta* position due to the stabilization of the phenoxyl radical by intramolecular hydrogen bonds [16,31,34]. Replacing a hydrogen atom with a methoxy group also increases the reducing capacity, but replacing an OH group with a methoxy group has the opposite effect [16,31,35,36]. Finally, hydroxycinnamic acids produce stronger signals than hydroxybenzoic acids due to the resonance stabilization of the radical, which improves the ability to donate protons [31,37].

To determine the influence of phenolic structures and the total polyphenol content on the outcome of FC assay results in more detail, we selected 24 different standard references representing the phenolic acids, flavonols, flavanols, dihydrochalones and flavanones. We investigated the potential of a new classification system based on reducing capacity. We also compared the results of the FC assay to other SET-based methods to determine which assay correlates most closely with the Bors criteria.

2. Materials and Methods

Chemicals and reference standards were obtained from Sigma-Aldrich (Steinheim, Germany): caffeic acid (CAA), (+)-catechin (CAT), 3,4-dihydroxybenzoic acid (DBA), (−)-epicatechin (EPC), ferulic acid (FEA), gallic acid (GAA), 4-hydroxybenzoic acid (HBA), hesperetin (HES), kaempferol (KAE), morin (MOR), naringenin (NAN), p-coumaric acid (PCA), phloridzin (PHD), phloretin (PHT), quercetin-3-D-galactoside (QGA3), quercetin-3-D-glucoside (QGU3), quercetin-7-D-glucoside (QGU7), quercetin (QUR), sinapic acid (SIA), siringic acid (SRA), taxifolin (TAF) and FC reagent. The standard reference narirutin (NAR) was obtained from K&J Scientific (Marbach am Neckar, Germany) and isorhamnetin (IRT) and naringin (NAG) from Carl Roth (Karlsruhe, Germany). Stock solutions were prepared by dissolving the reference standards in analytical grade absolute ethanol and diluting each of them in seven steps for the measurements.

The reducing capacity was determined by spectrophotometry as orignally described [28] with slight modifications. Briefly, 20 µL of each sample (reference standard dilution or ethanol) was mixed with 100 µL demineralized water (H_2O_{demin}) and 100 µL FC reagent and were incubated for 3 min in the dark. We then added 1580 µL of H_2O_{demin} and 200 µL 7.5% (w/v) sodium carbonate and incubated for another 30 min in the dark. The absorption of the samples was measured at 765 nm using a Specord 210 plus spectrophotometer (Analytik Jena, Jena, Germany). The slope was determined dy linear regression and presented as the reducing capacity.

For statistical analysis, Sigma Plot (Systat Software, San Jose, CA, USA) was used for one-way analysis of variance (ANOVA) corresponding to an unpaired t-test. If there was a significant difference, an additional pairwise test was carried out using the Holm–Šidák method. The significance level for both tests was 0.05. Statistical analysis was always carried out with all significant decimal places.

3. Results and Discussion

3.1. Analysis of Individual Reference Standards in the FC Assay

Phenolic reference compounds representing five different subgroups, which are shown in Table 1, were tested in the FC assay and the absorption of the reduced complex was quantified by spectrophotometry. The reducing capacities of all the reference compounds (calculated from the absorbance readings) are shown in Figure 2. The ranking was partly in agreement with previous studies [31,38–40].

The comparison of phenolic acids showed significantly different results for most of the compounds, with the exception of HBA and SRA as well as DBA and PCA. The presence of a galloyl group had the strongest influence on the reducing capacity, explaining the top-ranking position of GAA. We also found that a hydroxycinnamic acid group is more important than a hydroxybenzoic acid group, probably because the former enhances resonance stabilization [31,35,41]. Accordingly, SIA achieved a higher reducing capacity than SRA, PCA a higher value than HBA, and CAA a higher value than DBA. Furthermore, a higher reducing capacity was observed for compounds with a catechol group instead of an OH group at position 4, explaining why CAA showed a higher reducing capacity than PCA and FEA, and why DBA and GAA achieved higher values than HBA. Finally, the reducing capacity was also increased by the presence of an additional methoxy group, which is why SRA achieved a slightly higher value than HBA, and SIA a higher value than FEA and PCA. A methoxy group at position 5 appeared to confer greater reducing capacity than one at position 3, explaining the higher reducing capacity of DBA compared to SRA, and FEA compared to CAA. Replacing a hydrogen atom with a methoxy group, which is an electron donor, can reduce the bond dissociation energy, promote electron transfer, and increase the reducing capacity [16,31,35,36].

Among the flavonols, QUR achieved the highest reducing capacity because it fulfills all three Bors criteria. QGU7 was significantly less active because the OH at position 7 is replaced by a glucoside. In contrast to phenolic acids, the catechol group on the B-ring (corresponding to Bors 1) had the strongest influence on the reducing capacity of flavonols, explaining the high values observed for QUR, QGU7, QGA3 and QGU3. Furthermore, reducing capacity was increased by the presence of an OH group rather than a sugar at position 3, explaining the higher value observed for QUR compared to QGU3 and QGA3. Replacing a hydrogen atom with an electron donating group increases the reducing capacity, but replacing an OH group with an less electron donating group, such as a sugar, has the opposite effect [42]. The presence of an OH group at position 4′ had a significant effect whereas an OH group at position 2′ had a negligible effect, which is why there was no significant difference between MOR and KAE. An additional methoxy group again enhanced the reducing capacity, which is why IRT achieved a higher value than KAE. QGU3 and QGA3 were not significantly different and we assume that the type of sugar residue does not influence the reducing capacity.

The presence of a catechol group (Bors 1) also appeared to exert the strongest influence on the reducing capacity of flavanones, explaining why TAF achieved a higher value than HES, NAR and NAN. TAF also fulfills Bors 3, which contributes to its top-ranking position (in contrast to the other substances), so more research is needed to determine the influence of this structure. Although HES has an additional methoxy group on the B-ring, its reducing capacity was not significantly different to that of NAN. An OH group in the *para* position therefore appears to exert a greater influence than one in the *meta* position with an additional methoxy group. The presence of an OH group at position 7 also had a strong influence, which is why the reducing capacity of NAN was significantly higher than for NAR. As stated above for the flavonols, the type of sugar residue appeared not to influence the reducing capacity, explaining the similar values observed for NAR and NAG.

In the dihydrochalcone subgroup, PHT achieved a significantly higher value than PHD reflecting the additional OH group at position 4′. No general conclusions could be drawn because we only tested two different compounds. Similarly we only tested two flavanols (structural isomers) and the reducing capacity was similar for both compounds.

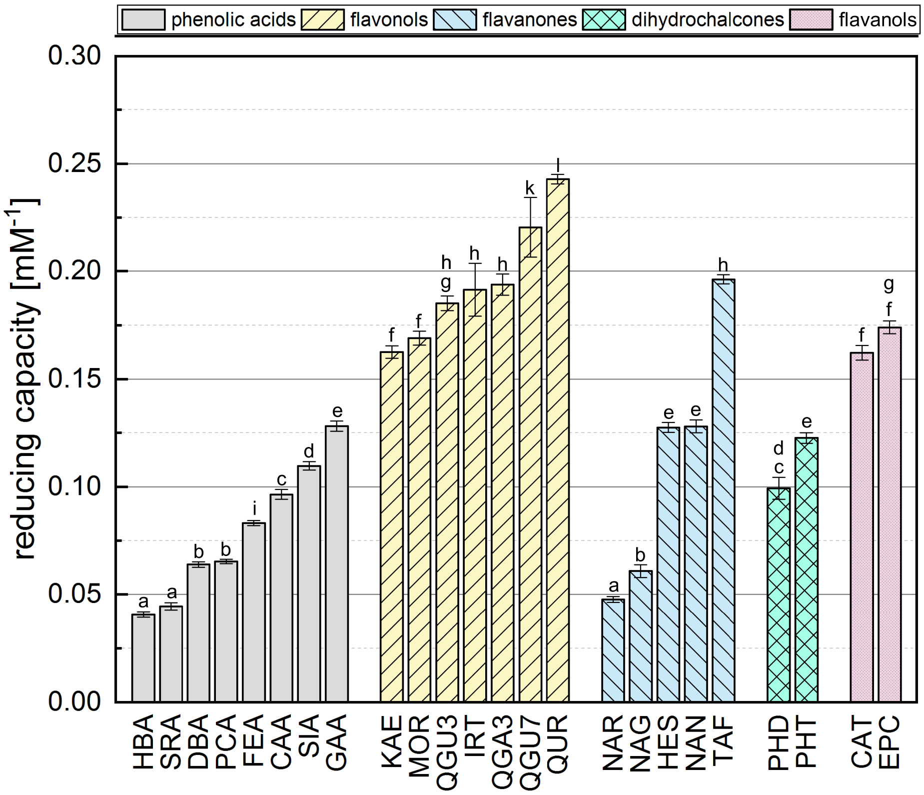


Figure 2. The reducing capacity of all standard reference compounds measured in the FC assay. Equal letters indicate that there is no significant difference between the values. The significance level was 0.05.

3.2. Consolidated Analysis of Phenolic Subgroups in the FC Assay

To gain insight into the general properties of the phenolic subgroups, we combined the individual assay results and evaluated the groups by mean reducing capacity as shown in Figure 3.

The phenolic acids, flavanones and dihydrochalcones showed the lowest mean values and there was no significant difference between them. This is probably because the compounds in these three categories feature the lowest number of OH groups (phenolic acids 1–3, flavanones 2–5, and dihydrochalcones 3–4) and most of them do not meet any of the Bors criteria (the only exception is TAF). The flavanols and flavonols showed higher reducing capacities and there was no significant difference between them. This was anticipated because they feature the highest number of OH groups (flavanols 5 and flavonols 4–5), all flavonols fulfill Bors 3, and some also fulfil Bors 1 and 2. Because the flavanols only fulfil Bors 1 yet are equivalent in value to the flavonols, we assume Bors 1 has the greatest influence on reducing capacity.

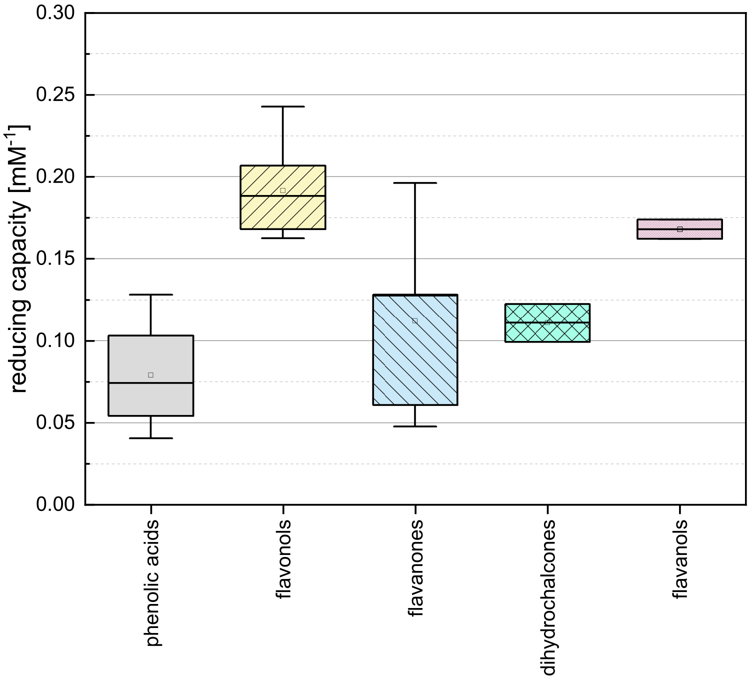


Figure 3. Boxplot of the mean reducing capacities of different subgroups of phenolic compounds (phenolic acids, flavonoles, flavanones, dihydrochalcones, flavanoles) in the FC assay. Error bars represent range within standard errors (1.5 interquartile range).

3.3. Reclassification of the Flavonoid Reference Standards Based on Their Structural Features

To evaluate the influence of particular structural features in more detail, we assigned the flavonoids reference standards to an alternative classification based on the number of OH groups and the satisfaction of Bors criteria (Table 2). The phenolic acids do not meet the Bors criteria because these were established only for flavonoids and therefore, we have not included them in our reclassification. We then combined the individual assay results and evaluated the new categories by mean reducing capacity as shown in Figure 4.

Table 2. Reclassification of flavonoids based on their structural features.

Class	Structural Feature	Substances
1	2 OH groups and none of the Bors criteria	NAG, NAR
2	3 to 4 OH groups and none of the Bors criteria	HES, NAN, PHD, PHT
3	one of the Bors criteria	CAT, EPC
4	two of the Bors criteria	IRT, KAE, MOR, QGA3, QGU3, TAF
5	three Bors criteria	QUR, QGU7

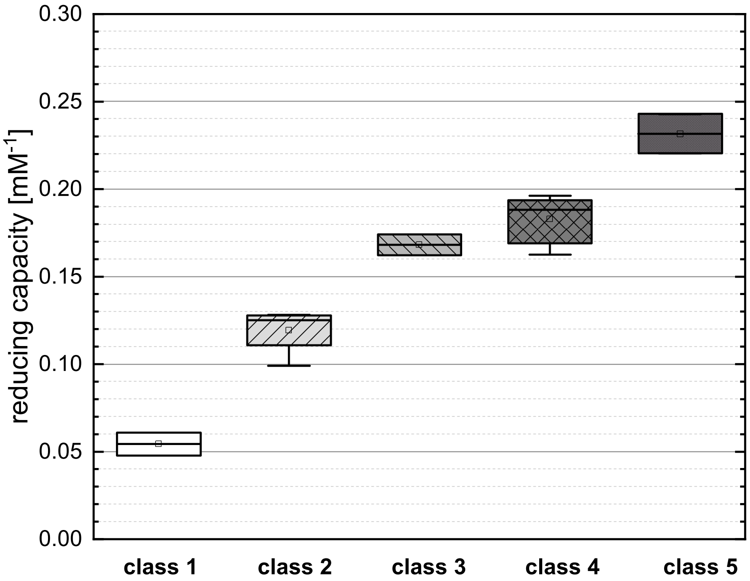


Figure 4. Boxplot of the mean values of the reducing capacity for the new classification of flavonoids as shown in Table 2, investigated in the FC assay. Error bars represent range within standard errors (1.5 interquartile range).

Class 1 (NAR and NAG) showed the lowest mean value in the boxplot, reflecting the low number of OH groups and the failure to meet any of the Bors criteria. Both substances presented similar values for reducing capacity, suggesting that the type of sugar at position 7 does not have a major influence. In class 2 (PHD, PHT, HES and NAN), PHD and NAN possess three OH groups but do not meet any Bors criteria and their reducing capacities were therefore low. The open-chain structure of PHD and PHT also appears to limit their reducing capacity. PHT and HES achieved similar values, so the position of the OH group appears to play only a minor role. We observed no significant difference between the two representatives of class 3 (the structural isomers CAT and EPC), indicating that the spatial arrangement of the molecule does not affect its reducing capacity. There were no significant differences between the substances in class 4 (KAE, MOR, QGU3, QGA3, IRT and TAF). All of the compounds fulfilled two of the three Bors criteria but not always the same two, so the effect of fulfilling the Bors criteria seems to be nonspecific and additive in this case. Furthermore, there was no significant difference between substances with different numbers of OH groups. Finally, class 5 (QGU7 and QUR) showed the highest mean value in the boxplot because both reference compounds fulfilled all three Bors criteria and also featured the highest number of OH groups.

3.4. Comparison of the FC Assay with the DPPH and ABTS Assays

Although some authors have reported a good correlation between the results of different in vitro antioxidant assays, others have reported significant discrepancies [21,23–26,43]. We therefore compared our FC results to our previously reported ABTS and DPPH assay data (Figure 5), where we used the same concentrations, temperature and measurement duration [15]. We observed no clear correlation between the assays, and the antioxidant activity seemed to depend on different criteria in each case. In contrast to the ABTS assay, the FC and DPPH assays appeared to depend mainly on the number of OH groups and the Bors criteria, with Bors 1 showing the greatest influence. The highest reducing capacity was observed for compounds meeting all three Bors criteria, which was not the case in the ABTS assay. However, an OH group in the *para* position of the B-ring appeared to play a greater role than the *meta* or *ortho* position in all three assays.

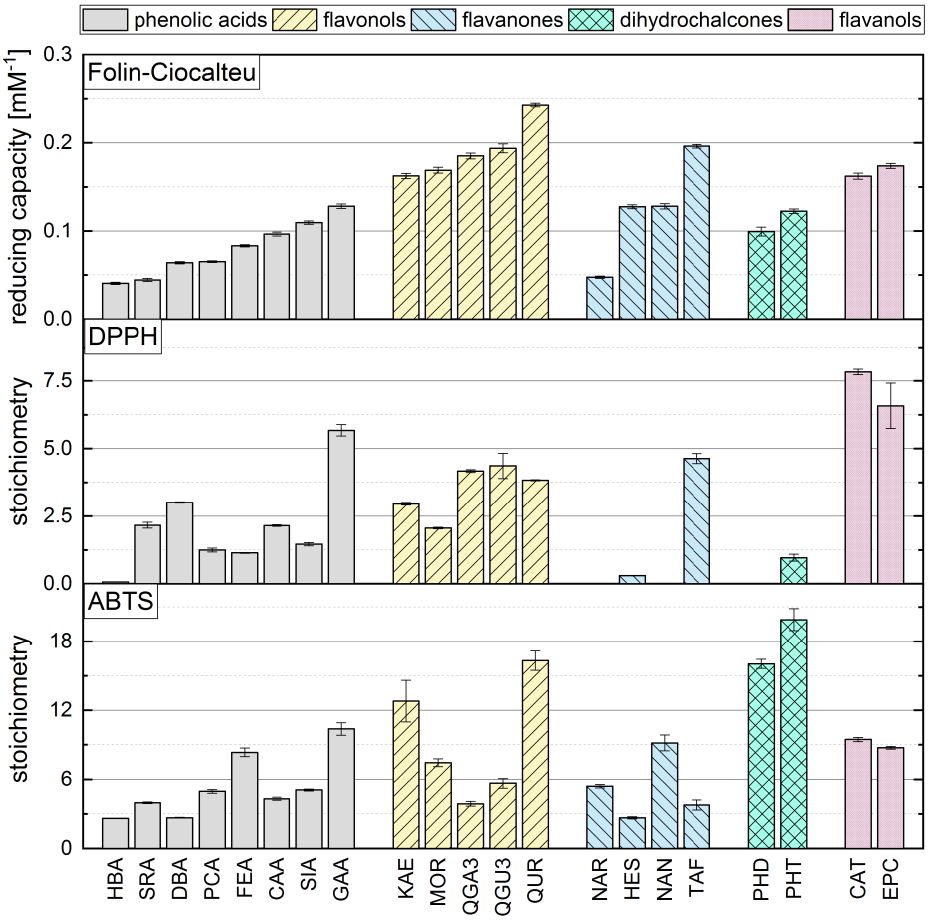


Figure 5. The reducing capacity of all standard references compounds measured in the FC assay in comparison to DPPH and ABTS assays, which are reproduced with permission from Platzer et al. (2021) [15].

Among the phenolic acids, the hydroxycinnamic acids achieved higher values than the hydroxybenzoic acids in the FC and ABTS assays, but not in the DPPH assay. Furthermore, substances with an additional methoxy group achieved higher values in all three assays. In the ABTS assay, reference compounds with OH and methoxy groups achieved even higher values than compounds with a catechol group, which was not the case in the FC and the DPPH assays. However, the outcome of the DPPH and FC assays depended mainly on the number of OH groups, whereas the presence of a galloyl group determined the result of the ABTS assay regardless of whether one or two OH groups was present. The DPPH and FC assay results for flavonols depended mainly on the Bors criteria. Substances in this subgroup always satisfy Bors 2, so the influence of Bors 1 and 3 should be considered. Bors 1 had the greatest influence in the DPPH and FC assays but not the ABTS assay. If a galloyl group replaces a catechol group on the B-ring, this only increased the value in the DPPH assay. In contrast, Bors 3 seemed to influence all three assays, because substances with a sugar residue at position 3 achieved lower values but the type of sugar was not important. Furthermore, an additional OH group in *ortho* position on the B-ring did not affect the result of any of the assays.

Bors 1 and 2 play a key role in all three assays when applied to flavanones. Furthermore, an OH group in the *para* position of the B-ring also increases the reducing capacity. The presence of a sugar residue had no influence on the FC assay and slightly worsened the value reported in the ABTS assay but inhibited the reaction in the DPPH assay.

For the dihydrochalcones, PHT achieved a higher value than PHD in all assays, reflecting the presence of an additional OH group. PHD did not react with the radical in the DPPH assay. There was no significant difference between the structurally isomeric flavanols CAT and EPC in any of the assays, indicating that the three-dimensional arrangement of these molecules does not influence the outcome.

4. Conclusions

The standard reference compounds measured in the FC assay produced significantly different values, indicating (as previously suggested) that the assay measures reducing capacity rather than the total polyphenol content. The ranking of reference compounds in the FC assay was partly in agreement with the literature and suggested that the reported reducing capacity depends on the SAR, as determined by the number and position of OH groups as well as the satisfaction of Bors criteria. By assigning the reference compounds to five newly-defined classes, we found that the results of the FC assay depend primarily on how many of the Bors criteria are met, with the number of OH groups becoming relevant only if the molecule fails to fulfill any of the Bors criteria. Correlation with other SET-based assays (ABTS and DPPH) is often reported in literature, but we found no clear relationship between them. Interestingly, the three assays appeared to depend (at least in part) on the same structural features, but with different outcomes. One feature common to all three assays was the greater influence of an OH group in the *para* position rather than the *meta* position. The FC assay also appeared to correlate better with the Bors criteria, and thus the SAR of the antioxidants, compared to the other two assays. Accordingly, the antioxidant effect of phenolic compounds in the FC assay can be predicted based on their structural characteristics. Furthermore, all the reference compounds could be detected in the FC assay, whereas the DPPH assay did not show a reaction for some flavanones and dihydrochalcones. Therefore, the FC assay should be preferred especially for extracts rich in these substances. In order to compare the results of the FC assay with the antioxidant activity in specific applications, a correlation in the final medium (e.g., plant oil or food matrix) should be clarified. In addition, other relevant parameters such as pH, solvent, the content of other components (e.g., sugars and proteins) and synergistic effects between different phenolic compounds should be considered when extracts are measured, as these may influence the reported activity. These aspects require more detailed analysis in future experiments.

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Abbreviations

The following abbreviations are used in this manuscript:

ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
CAA	caffeic acid
CAT	(+)-catechin
DBA	3,4-dihydroxybenzoic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EPC	(−)-epicatechin
FC	Folin-Ciocalteu
FEA	ferulic acid
GAA	gallic acid
HAT	hydrogen atom transfer
HBA	4-hydroxybenzoic acid
HES	hesperetin
IRT	isorhamnetin
KAE	kaempferol
MOR	morin
NAG	naringin
NAN	naringenin
NAR	narirutin
ORAC	oxygen radical absorbance capacity
PCA	*p*-coumaric acid
PHD	phloridzin
PHT	phloretin
QGA3	quercetin-3-D-galactosides
QGU3	quercetin-3-D-glucosides
QGU7	quercetin-7-glucoside
QUR	quercetin
SIA	sinapic acid
SAR	structure-activity relationship
SET	single electron transfer
SRA	siringic acid
TAF	taxifolin

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4.3 Radical scavenging mechanisms of phenolic compounds: A quantitative structure-property realtionship (QSPR) study [3]

Summary

In addition to the SET-based *in vitro* antioxidant assays, HAT-based assays, such as the ORAC, are also used to measure antioxidant activity. Since not much is known in the literature about the structure-activity relationships of this assay and the correlation to SET-based assays, these topics were addressed in this study. Again, the same 24 standard references were analyzed to identify structural properties leading to a high antioxidant capacity in the ORAC assay. Furthermore, the results were compared to those from ABTS, DPPH and FC assays to investigate common trends and differences regarding structural properties leading to a high antioxidant behavior.

In this study, it was shown that the results in the ORAC assay depended particularly on the number of hydroxyl groups of a molecule and less on their structural arrangement on the molecule. High activities resulted for substances with a catechol group. The Bors criteria, with the exception of Bors 2, played a minor role for the result in the ORAC assay. By statistical evaluation using principle component (PC) analysis, it was possible to establish correlations between the different *in vitro* antioxidant assays. According to these analyses, the ORAC and ABTS assay were influenced by different structural properties than the DPPH and FC assay, which indicated different reaction mechanisms. It was also found that each of the assays showed a strong dependence on antioxidant behavior, implying that, in principle, each of the four assays is suitable for the determination of antioxidant activity. Furthermore, the number of hydroxyl groups was found to have the highest influence on the antioxidant behavior whereas the Bors criteria only partially influenced the antioxidant behavior. It was also shown that the DPPH assay was suitable to measure flavanol-rich samples, while the ABTS and ORAC assay should be used for dihydrochalcone-rich and non-glycosylated flavonols, respectively.

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[3] Platzer M., Kiese S., Tybussek, T., Herfellner T., Schneider F., Schweiggert-Weisz U., Eisner P., Radical scavenging mechanisms of phenolic compounds: A quantitative structure-property relationship (QSPR) study, *Frontiers in Nutrition* **9**, 663 (2022)

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- Melanie Platzer: conception and design of the study, methodology, data curation, visualization, statistical analysis and writing-original draft preparation
- Sandra Kiese: conception and design of the study, methodology, data curation, visualization and supervision
- Thorsten Tybussek: methodology, data curation and visualization
- Thomas Herfellner: writing-review and editing, supervision and funding acquisition
- Franziska Schneider: writing-review and editing
- Ute Schweiggert-Weisz: writing-review and editing and supervision
- Peter Eisner: validation and writing-review and editing and supervision

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Radical Scavenging Mechanisms of Phenolic Compounds: A Quantitative Structure-Property Relationship (QSPR) Study

Melanie Platzer[1,2]*, Sandra Kiese[2], Thorsten Tybussek[2], Thomas Herfellner[2], Franziska Schneider[2], Ute Schweiggert-Weisz[2,3] and Peter Eisner[1,2,4]

[1] TUM School of Life Sciences Weihenstephan, ZIEL-Institute for Food & Health, Technical University of Munich, Freising, Germany, [2] Fraunhofer Institute for Process Engineering and Packaging IVV, Freising, Germany, [3] Chair of Food Science, Institute for Nutritional and Food Sciences, University of Bonn, Bonn, Germany, [4] Faculty of Technology and Engineering, Steinbeis-Hochschule, Dresden, Germany

Due to their antioxidant properties, secondary plant metabolites can scavenge free radicals such as reactive oxygen species and protect foods from oxidation processes. Our aim was to study structural influences, like basic structure, number of hydroxyl groups and number of Bors criteria on the outcome of the oxygen radical absorbance capacity (ORAC) assay. Furthermore, similarities and differences to other *in vitro* antioxidant assays were analyzed by principal component analysis. Our studies confirmed that the antioxidant behavior in the ORAC assay is dominated by the number and types of substituents and not by the Bors criteria, as long as no steric hindrance occurs. For example, morin (MOR) with five hydroxyl groups and two Bors criteria reached an area under the curve of (3.64 ± 0.08) $\times 10^5$, which was significantly higher than quercetin-7-D-glucoside (QGU7) ($P < 0.001$), and thus the highest result. Principal component analysis showed different dependencies regarding structural properties of Folin-Ciocalteu (FC)- and 2,2-diphenyl-1-picrylhydrazyl (DPPH)-assays or 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)- and ORAC-assays, respectively. Therefore, we conclude that they are based on different reaction mechanisms. The number of hydroxyl groups showed a stronger influence on the antioxidant activity than the Bors criteria. Due to these differences, the correlation of these rapid tests to specific applications should be validated.

Keywords: area under the curve, antioxidant effect, flavonoids, phenolic acids, structure-activity relationship

Edited by:
Fuguo Liu,
Northwest Aand F University, China

Reviewed by:
Viduranga Y. Waisundara,
Australian College of Business and Technology, Sri Lanka
Kin Weng Kong,
University of Malaya, Malaysia

***Correspondence:**
Melanie Platzer
melanie.platzer@ivv.fraunhofer.de

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1. INTRODUCTION

Secondary plant metabolites are responsible for various functions in plants, such as protection against herbivores, UV radiation, pests and pathogens (1–3). Since these compounds are found more abundantly in the edible parts of plants and are also known for their health-promoting and disease-preventing properties, they are of particular interest to the food and pharmaceutical industries. Due to their antioxidant properties, they can scavenge free radicals such as reactive oxygen species and protect foods from oxidation processes, significantly improving their storage stability and quality (1, 4–8). Phenolic compounds, which are prominent representatives of secondary plant metabolites, are among the most important naturally occurring antioxidants

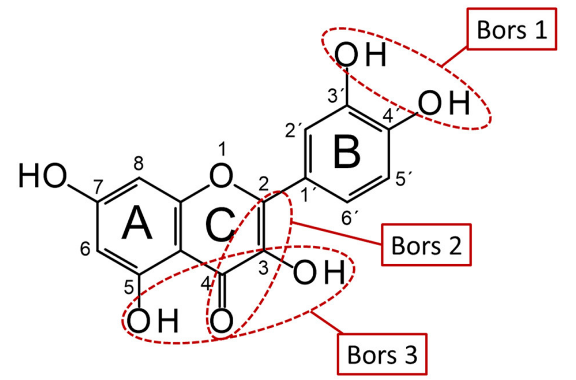

FIGURE 1 | Schematic representation of Bors criteria using QUR. Bors 1: catechol group on the B-ring; Bors 2: double bond between C-2 and C-3 and a carbonyl group at C-4 on the C-ring; Bors 3: hydroxyl groups at C-3 and C-5 on the A- and C-rings and 4-oxo group on the C-ring (reproduced from Platzer et al. (11)).

and can be classified into different subgroups based on their structural properties. In addition to low-molecular compounds such as phenolic acids, there are several, more complex representatives including the group of flavonoids (9, 10). The strength of the antioxidant effect of these compounds has been described in numerous publications and depends on their structural properties, such as the Bors criteria (**Figure 1**) (11–14).

Analytical methods, such as the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC) or Folin-Ciocalteu (FC) assays are often used to measure the antioxidant activity (15–18). These rapid tests can be distinguished based on the underlying reaction mechanism, the single electron transfer (SET) and the hydrogen atom transfer (HAT), which run both in parallel, but at different rates (19–24).

SET-based assays, such as ABTS, DPPH, and FC (18, 19, 25–27), measure the electron loss of a free radical ($R^\cdot$) resulting in a radical anion and the reaction mechanisms were described elsewhere (11, 14). In the HAT-based mechanism (Reaction 1) a hydrogen atom is delivered from the antioxidant (AOH) directly to a radical to interrupt the oxidative chain reaction.

$$R^\cdot + AOH- > RH + AO^\cdot \qquad (1)$$

The ORAC assay is a popular example of a HAT-based assay and is particularly used in the food industry (18, 19, 25–27). In contrast to the ABTS, DPPH, and FC assays, the measurement principle is not based on a color loss or color change, but on a decrease in fluorescence (19, 28–30). The presence of antioxidants neutralizes the peroxyl radicals by either a hydrogen atom transfer or a radical addition, which results in the fluorescence decrease being slowed down (30, 31). This decrease is measured as a function of time and evaluated subsequently as area under the curve (AUC) (30, 31). The AUC represents the antioxidant effect of a substance and, in contrast to other evaluation methods, combines the inhibition time and the amount of free radicals neutralized by antioxidants (32–34). Therefore, the test is suitable for antioxidants with or without a pronounced lag phase, which is particularly useful for food samples that often consist of several different ingredients (30, 35). Furthermore, the assay is suitable for both hydrophobic and hydrophilic substances (31). Although the ORAC assay is a widely used method, little is known about structural properties that have an impact on the measurement results. In the literature, there are mainly theoretical studies (20, 23, 24, 36–43) on the commonalities of the two reaction mechanisms and less systematic experimental studies on the correlations of different assays in terms of influence of the basic structure, number of hydroxyl groups and number of fulfilled Bors criteria. In addition, not much is reported on whether the same structural properties are crucial for SET- and HAT-based assays and how they differ from each other.

Therefore, we investigated the influence of structural properties of phenolic compounds on the outcome of the ORAC assay. For this purpose, different standard references belonging to the subgroups of phenolic acids, flavonols, flavanones, dihydrochalcones, and flavanols were analyzed. Furthermore, we compared these results to those from ABTS, DPPH and FC assays to investigate common trends and differences regarding structural properties leading to high antioxidant behavior. To investigate this in more detail, the results were clustered according to different criteria and presented in a principal component analysis. Special attention was paid to the subgroup, the number of hydroxyl groups, the number of fulfilled Bors criteria as well as the reaction mechanism in the respective assay.

2. MATERIALS AND METHODS

Chemicals and reference standards (see **Table 1** slightly modified from **Table 1** in Platzer et al. (11, 14)) were obtained from Sigma-Aldrich (Steinheim, Germany): 2,2'-azubis-(2'-methylpropionamidine) dihydrochloride (AAPH), caffeic acid (CAA), (+)-catechin (CAT), 3,4-dihydroxybenzoic acid (DBA), (−)-epicatechin (EPC), ferulic acid (FEA), fluorescein, gallic acid (GAA), 4-hydroxybenzoic acid (HBA), hesperetin (HES), kaempferol (KAE), morin (MOR), myricetin (MYR), naringenin (NAN), *p*-coumaric acid (PCA), phloridzin (PHD), phloretin (PHT), quercetin-3-D-glucoside (QGU3), quercetin-7-glucoside (QGU7), quercetin (QUR), sinapic acid (SIA), syringic acid (SRA), taxifolin (TAF) and Folin-Ciocalteu (FC) reagent. The

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Abbreviations: AAPH, 2,2'-azubis-(2'-methylpropionamidine) dihydrochloride; ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); AOH, antioxidant; AUC, area under the curve; CAA, caffeic acid; CAT, (+)-catechin; DBA, 3,4-dihydroxybenzoic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EPC, (−)-epicatechin; FC, Folin-Ciocalteu; FEA, ferulic acid; GAA, gallic acid; HAT, hydrogen atom transfer; HBA, 4-hydroxybenzoic acid; HES, hesperetin; IRT, isorhamnetin; KAE, kaempferol; MOR, morin; MYR, myricetin; NAG, naringin; NAN, naringenin; NAR, narirutin; ORAC, oxygen radical absorbance capacity; PCA, p-coumaric acid; PHD, phloridzin; PHT, phloretin; QGU3, quercetin-3-D-glucoside; QGU7, quercetin-7-glucoside; QUR, quercetin; SIA, sinapic acid; SET, single electron transfer; SRA, syringic acid; TAF, taxifolin.

standard reference narirutin (NAR) was obtained from K&J Scientific (Marbach am Neckar, Germany) and isorhamnetin (IRT) and naringin (NAG) from Carl Roth (Karlsruhe, Germany). Stock solutions were prepared by dissolving the reference standards in analytical grade ethanol$_{\text{absolute}}$ and diluting each of them in seven steps (2E-4 to 1E-1 mM) for the measurements.

The AUC was determined using the ORAC assay according to the procedure of Huang et al. (44) with slight modifications. The stock solution of fluorescein was prepared by dissolving 1.33 mg in 100 ml 75 mM phosphate buffer and stored in the dark at 4 °C. The working solution, which was made freshly every day, was prepared by diluting the stock solution 1:1,000 with 75 mM phosphate buffer. For the AAPH solution, which was also freshly prepared daily, we dissolved 414 mg in 10 ml of the 75 mM phosphate buffer and stored the solution at 4°C until use. For the measurement, a 96-well plate was used, where the outer cells were filled with water. 25 µl of each sample together with 150µl of fluorescein stock solution were added to the wells and incubated for 30 min at 37°C in a microplate reader (Infinite 230 PRO, TECAN (Männedorf, Switzerland). The reaction was started by addition of 25 µl AAPH solution and fluorescence was recorded every 60 s, after shaking the plate for 10 s, as kinetics over time (excitation wavelength 485 nm, 20 nm bandpass, emission wavelength 535 nm, 20 nm bandpass). Each sample was measured in triplicate.

The raw data were evaluated following Cao and Prior (45) with some modifications. The obtained curves were normalized to their initial value and subsequently integrated to obtain the AUC. Net AUC (AUC_{sample} - AUC_{blank}) was plotted as a function of AOH concentration. To calculate the value of a 1 mM solution, a linear regression equation was used. In order to not underestimate the AUC, only the concentrations below saturation were used as already described in Platzer et al. (14).

The results from the ABTS, DPPH and FC assays are shown here for comparison only and do not represent new results. Therefore, the corresponding method descriptions can be found in the literature and are not repeated here (11, 14).

For statistical analysis, a one-way analysis of variance (ANOVA) was performed using Sigma Plot (Systat Software, San Jose, CA, USA), equivalent to an unpaired *t*-test. If a significant difference was found, an additional paired test using the Holm-Šídák method was added. The significance level for both tests was at $P < 0.05$. Statistical analysis was always performed with all significant decimal places. The performance of the statistical analyzes was carried out for all measured substances. To investigate significant differences within the phenolic subgroups, the statistical analysis was additionally performed within the respective group and reference was included at the corresponding positions in the text. A multivariate analysis was performed by principal component analysis using OriginPro2018.

3. RESULTS AND DISCUSSION

Table 1 shows the phenolic compounds used in this study and their five different subgroups (slightly modified from Table 1 in Platzer et al. (11, 14)).

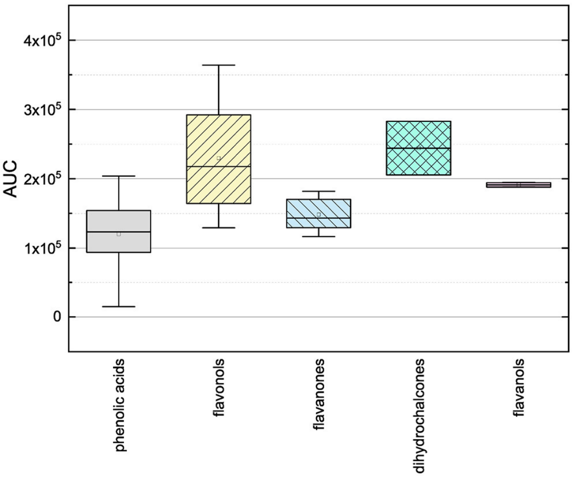

FIGURE 2 | Boxplot of the mean values of AUC of different subgroups of phenolic compounds (phenolic acids, flavonoles, flavanones, dihydrochalcones, flavanoles) in the ORAC assay. The range within standard errors (1.5 interquartile range) is represented by error bars.

3.1. Consolidated Analysis of Phenolic Subgroups in the ORAC Assay

In order to investigate the antioxidant behavior of the phenolic subgroups, the results of each subgroup were averaged and are presented in **Figure 2** as boxplot.

The phenolic acids, the flavanones and the flavanols obtained lower mean values and the flavonols and dyhydrochalcones slightly higher mean values. The low mean values of the phenolic acids and the flavanones could be explained by their low number of hydroxyl groups (phenolic acids 1–3, flavanones 2–5). The substances used, with exception of TAF, do not meet any Bors criteria. The flavanols also reached a low mean value, but it should be noted that only two substances were measured in this group. As those two substances have five hydroxyl groups and fulfill the first Bors criterion, a higher value was expected. The dihydrochalcones having three or four hydroxyl groups reached the second highest mean value. Here, a lower value was expected because the substances do not fulfill any of the Bors criteria and also have a lower number of hydroxyl groups than the flavanols. The flavonols reached the highest mean value, which could be explained by their high number of hydroxyl groups (4–6) and by the fact that most of the Bors criteria are fulfilled. Thus, the result in the ORAC assay seems to depend on both the number of hydroxyl groups and the fulfilled Bors criteria. In order to analyze this influence in more detail, the results were rearranged and are discussed later (see Section 3.3).

The mean values of the substances that do not fulfill any of the Bors criteria is not significantly different from those that fulfill one, two or three criteria. Considering the influence of the number of hydroxyl groups on the molecule, the mean value increased with increasing number of hydroxyl groups. However, substances with five hydroxyl groups partially showed a lower antioxidant behavior, with the exception of MOR, which also

TABLE 1 | Phenolic compounds analyzed in this study (phenolic acids and subgroups of flavonoids) along with reference standard names, sample codes and substituents (reproduced and slightly modified from this table in Platzer et al. (11, 14)).

Subgroups	Reference standards	Code	Position and substituents						
Phenolic acids			**1**	**3**	**4**	**5**			
	Caffeic acid	CAA	$(CH_2)_2COOH$	OH	OH	H			
	3,4-dihydroxybenzoic acid	DBA	COOH	OH	OH	H			
	Ferulic acid	FEA	$(CH_2)_2COOH$	OCH_3	OH	H			
	Gallic acid	GAA	COOH	OH	OH	OH			
	4-hydroxybencoic acid	HBA	COOH	H	OH	H			
	p-coumaric acid	PCA	$(CH_2)_2COOH$	H	OH	H			
	Sinapic acid	SIA	$(CH_2)_2COOH$	OCH_3	OH	OCH_3			
	Syringic acid	SRA	COOH	OCH_3	OH	OCH_3			
Flavonols			**2’**	**3’**	**4’**	**5’**	**3**	**5**	**7**
	Isorhamnetin	IRT	H	OCH_3	OH	H	OH	OH	OH
	Kaempferol	KAE	H	H	OH	H	OH	OH	OH
	Morin	MOR	OH	H	OH	H	OH	OH	OH
	Myricetin	MYR	H	OH	OH	OH	OH	OH	OH
	Quercetin-3-D-glucoside	QGU3	H	OH	OH	H	Gal	OH	OH
	Quercetin-7-D-glucoside	QGU7	H	OH	OH	H	OH	OH	Glc
	Quercetin	QUR	H	OH	OH	H	OH	OH	OH
Flavanones			**3’**	**4’**	**3**	**5**	**7**		
	Hesperetin	HES	OH	OCH_3	H	OH	OH		
	Narirutin	NAR	H	OH	H	OH	2 Glc		
	Naringin	NAG	H	OH	H	OH	Rham, Glc		
	Naringenin	NAN	H	OH	H	OH	OH		
	Taxifolin	TAF	OH	OH	OH	OH	OH		
Dihydrochalcones			**4**	**2’**	**4’**	**6’**			
	Phloridzin	PHD	OH	OH	OH	Glc			
	Phloretin	PHT	OH	OH	OH	OH			
Flavanols			**3’**	**4’**	**3**	**4**	**5**	**7**	
	(+)-catechin	CAT	OH	OH	OH	H	OH	OH	
	(−)-epicatechin	EPC	OH	OH	OH	H	OH	OH	

explains the outlier in the graph. MYR, which has six hydroxyl groups showed a significantly lower value. From these results we assume that steric hindrance could lead to a reduction of the antioxidant potential.

To analyze the structural properties of the phenolic compounds in more detail, the individual results of each substance are discussed in the following.

3.2. Analysis of Individual Reference Standards in the ORAC Assay

The AUCs of all reference standards, divided into their subgroups, are shown in **Figure 3**. The results of the significance analysis using all reference standards are shown by squares in **Figure 4**. The ranking of reference standards was partly in agreement with previous studies (39, 46–49). In the following, the results of the individual subgroups are discussed, which is why the significance test was performed additionally within the respective group, as shown by circles in **Figure 4**.

When comparing the results of the phenolic acids, similar values were found, which did not differ significantly from each other. The presence of a catechol group had the strongest influence, which is why CAA and DBA achieved the highest values. PCA and HBA, which both have only one hydroxyl group, achieved lower values, which is consistent with literature (48, 50). The presence of a catechol group confers more stability to the B-ring by participating in electron delocalization thereby increasing the antioxidant activity (36, 43, 51–53). Due to resonance stabilization, *para-meta* hydroxylated substances exhibit enhanced antioxidant activity compared to monohydroxylated substances (50). However, if the molecule possesses a hydroxyl group in addition to the catechol group (galloyl group), this has a negative effect on the antioxidant activity in the ORAC assay, which is why GAA reached the lowest value and was partially in agreement with literature (54, 55). A lower value for a substance with a galloyl group compared to the catechol group was also shown in other studies, even if a different result was expected, due to the three hydroxyl groups on the aromatic ring (39, 54, 56–60). This can probably be attributed to steric hindrance.

The presence of additional methoxy groups to a hydroxyl group also leads to a lower value, which is why SIA, FEA and SRA achieved lower values than HBA and PCA. Again, steric

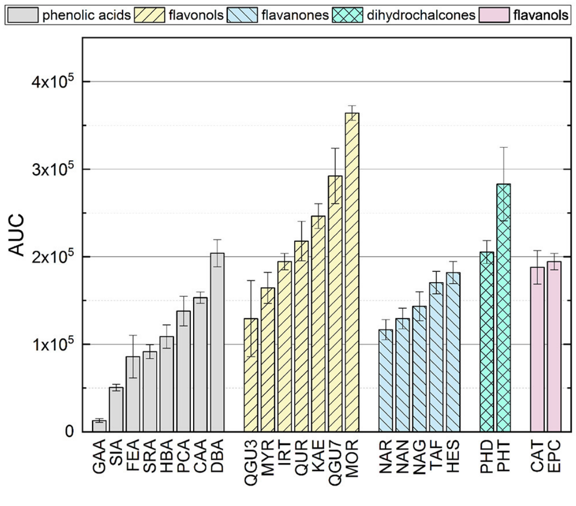

FIGURE 3 | AUC of all standard reference compounds determined by ORAC assay.

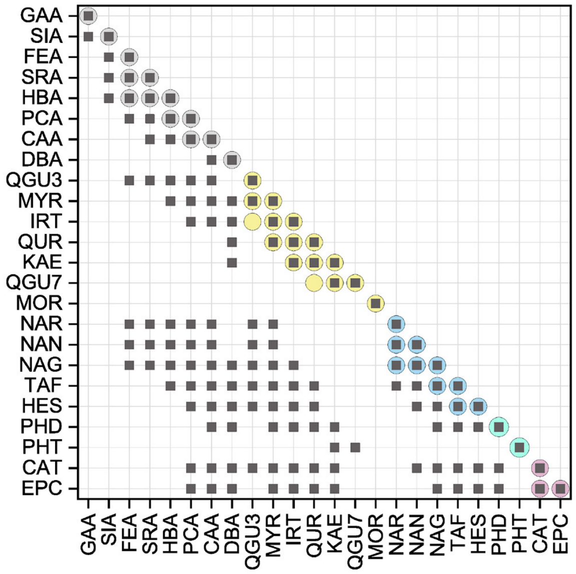

FIGURE 4 | Results of the statistical analysis of the ORAC results with a significance level at $P < 0.05$. Squares indicate the results, which were not significantly different ($P > 0.05$) using all standard references. Circles indicate the results of the statistical analysis within the subgroups, which were not significantly different ($P > 0.05$).

hindrance is the propable cause for these results, just as with GAA, SIA, which also have three substituents, obtained a lower value than FEA. This has also been shown in other studies (39, 56–60). Furthermore, the replacement of a hydroxyl group by a methoxy group leads to a lower antioxidant activity, which can be explained by the fact that methylation leads to a decrease in active electron- and hydrogen-donating groups (50, 58, 61–63). In contrast to the hydroxycinnamic acids, the hydroxybenzoic acids have a higher antioxidant effect, which is why DBA and SRA achieved higher values than CAA and SIA. This result was expected to be different, because the additional conjugated double bond between the benzene ring and the carboxyl group leads to extended electron delocalization and should stabilize the resulting radical (50, 58, 61–63). HBA and PCA are exceptions here, as they do not differ significantly.

In contrast to the phenolic acids, the presence of a catechol group on the B-ring for the flavonols did not have the highest influence on the result. In their case, the substitution of the B-ring plays a minor role, contrary to what might be assumed from the first Bors criterion. KAE with only one hydroxyl group on the B-ring did not obtain a significantly different result as QUR, which has a catechol group. This result was expected to be different, since the B-ring of flavonoids is considered to be the most reactive group and the delocalization of the unpaired electron preserves the stability of the phenoxyl radical. Moreover, an intramolecular hydrogen bond is formed by a catechol group, which should further enhance the π-delocalization in the B-ring (38, 43, 58, 64–66). MOR, which has two hydroxyl groups, but not a catechol group, obtained the highest result in this study. A possible explanation could be the rotation of the B-ring leading to a stabilization of the O-radical at C-2' by a intramolecular hydrogen bond with 3-OH (42, 67). MYR, which has a galloyl group on the B-ring, showed a lower value in comparison to KAE with only one hydroxyl group and no significant different result when compared to QUR. We assume that steric hindrance of the molecule may occur if the substituents are adjacent to each other, leading to a reduction of the antioxidant behavior of these compounds in the ORAC assay. In addition to hydroxyl groups, C/O-glycosides and O-methylation also have a negative influence on the results, as shown by some studies (39, 56–60). There was also a negative effect with IRT, KAE, and QUR, although it should be noted that an additional hydroxyl group instead of O-methylation does not improve the antioxidant behavior either (QUR=IRT). The work of Kang et al. (68) also showed lower values for substances that have a methoxy group instead of a hydroxyl group on the benzene ring. Different results were also obtained for O-glycosylation. Thus, no significant difference was obtained for the substances QGU7 and QUR. Therefore, the presence of a sugar residue at C-7 seems not to have any influence on the result. This does not apply for QGU3 and QUR, where the sugar at C-3 has a prooxidative effect. A hydroxyl group at C-3 supports the reaction with the free radical, which is further favored by the presence of a hydroxyl group at C-5 and C-7 by electron donating effect (69, 70). The presence of Bors 2 and a 3-OH group on the C-ring also affected the antioxidant behavior in previous studies (39, 71). The presence of both structural properties additionally benefits the electron delocalization increasing the antioxidant activity (58, 64–66).

Also the presence of a galloyl group had no influence on the result, which is why MYR and QUR also did not achieve a significantly different value, which is not consistent with literature (72). MOR, having two hydroxyl groups, obtained a higher value than KAE with only one hydroxyl group and QUR with a catechol group. This result is consistent with the studies of Promden et al. (73), who also showed that the presence of a hydroxyl group at 3' and 5' strongly increases the result in the ORAC. The presence of a hydroxyl group in *ortho* position results in stabilization of the phenoxyl radical due to intramolecular hydrogen bonding leading to increased antioxidant activity (58, 74, 75). The results are also partially in agreement with those of Žuvela et al. (37) and not in agreement with Cao et al. (47). Again, KAE showed a higher value than MYR and QUR, which was in agreement with the work of Ishimoto et al. (49). Thus, two hydroxyl groups on the B-ring seem to improve the result as long as they are not adjacent to each other. Furthermore, the result of the ORAC assay was not negatively influenced by a methoxy group at C-3' of the B-ring instead of a hydroxyl group, leading to similar results for IRT and QUR, which was also shown in literature (49). The influence of the second Bors criterion cannot be assessed on the basis of our measurements, since it is fulfilled by all substances used. The only substance that does not fulfill the third Bors criterion (QGU3) achieved the lowest value. Therefore, the third Bors criterion seems to be the decisive one. Additionally, a sugar residue in place of a hydroxyl group at C-7 of the A-ring had no influence on the AUC, which is why QGU7 and QUR achieved similar values.

For the flavanones, just as for the flavonols, the presence of a hydroxyl group at C-7 of the A-ring had no effect on the antioxidant activity, which is why NAN and NAG, as well as NAN and NAR did not show a significant difference in AUC. In contrast to the phenolic acids and the flavonols, substances with two substituents on the B-ring obtained the highest values. However, it does not seem to matter whether this is a hydroxyl or a methoxy group, which is why TAF and HES achieved similar results and HES had a higher value than NAN. One possible explanation is the increase of the electron accessibility of the carbon atoms in the aromatic ring leading to a high antioxidant behavior for electron donating groups such as a methoxy group in *para* position (76, 77). The influence of a hydroxyl group at C-3 of the C-ring is not clear from these results, since only TAF has this group. Since TAF achieved a higher value than NAN, it is possible that there is a positive influence. However, since the structures differ in other features, these could have an additional influence. Since HES reached a higher value than NAN, a hydroxyl group in *para*-position does not have any influence on the result in the ORAC assay, although this property is considered to promote an antioxidant behavior (11, 13, 14, 78, 79). The influence of a hydroxyl group at C-3 of the C-ring is also not clear from our results.

The results of the dihydrochalcones were significantly different from each other, with PHT obtaining a higher value than PHD. Thus, a hydroxyl group at C-6' of the A-ring instead of a sugar residue had a positive influence on the result.

There was no significant difference for the flavanols, which was expected since CAT and EPC are structural isomers and was also shown in literature (49).

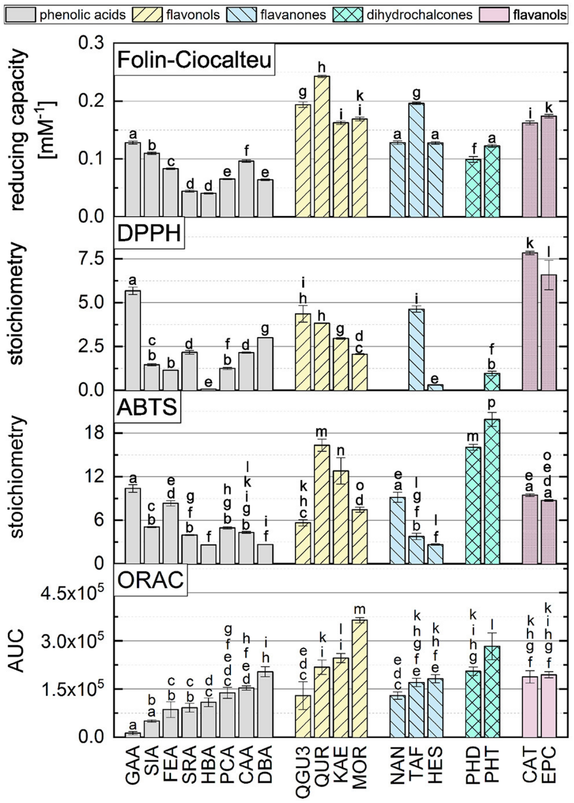

FIGURE 5 | The AUC of all standard reference compounds measured in the ORAC assay in comparison to FC, DPPH and ABTS assays, which are reproduced from Platzer et al. (11, 14). Equal letters indicate that there is no significant difference between the results with an significance level at $P > 0.05$.

3.3. Comparison of the ORAC Assay With the DPPH, ABTS and FC

According to literature, there are different results on the correlation of different antioxidant *in vitro* methods (11, 14, 18, 19, 25–27). To investigate this in more detail, our ORAC assay results and structure activity relationships are compared to three SET-based assays (ABTS, DPPH and FC), which were published previously by Platzer et al. (11, 14). The results of the individual standard references are presented in **Figure 5**. In the following section, we discuss similarities and differences of the influences of structural properties leading to high values in the ABTS, DPPH, FC and ORAC assays.

No clear correlation can be found, when comparing the results of the four assays. This suggests that the assays are influenced by different structural properties. According to literature, the antioxidant behavior depends on both, number and position of hydroxyl groups and other substituents of a molecule (13).

For the group of phenolic acids, all assays showed higher values for the hydroxycinnamic acids than for the hydroxybenzoic acids, with exception of the DPPH assay. In the ABTS, DPPH and FC assays, in contrast to the ORAC assay, the results were not increased by an additional methoxy group. In the ABTS assay, substances with one hydroxyl and one methoxy group showed even higher values than substances with one catechol group. An increasing number of hydroxyl groups, also increased the results of a phenolic acid in the DPPH and FC assays. In the ABTS and ORAC assays, substances with a galloyl group showed significantly lower values, which could be explained by steric hindrance. In some cases, substances with one or two hydroxyl groups did not show significantly different values. For the DPPH and FC assays, the Bors criteria play a crucial role especially in the group of flavonols, with the first Bors criterion having the strongest influence. This effect was not seen for the ABTS or the ORAC assay. Substances with a galloyl group instead of a catechol group only achieved higher values in the DPPH. Again, steric hindrance may reduce the antioxidant behavior in the other assays. In all four assays, the third Bors criterion had an influence on the results, which is why QUR achieved higher values than QGU3. Substances which have an additional hydroxyl group in *ortho* position showed an increased value only in the ORAC assay. In the case of the flavanones, the second and the third Bors criterion had a positive influence on the results in the ABTS, DPPH and FC assays and also partially in the ORAC assay. A hydroxyl group in *para* position increased the results in all assays, with exception of the ORAC assay. The influence of a sugar residue in the four assays was either negative or there was no influence on the results. For the dihydrochalcones, PHT reached a higher value than PHD in all four assays, which can be explained by the additional hydroxyl group. PHD showed no reaction in the DPPH assay. For the two structural isomers EPC and CAT, which belong to the group of flavanols, similar values were reached in all four assays and it can be concluded that the three-dimensional arrangement of the molecules has no influence on the reaction in the *in vitro* methods (11, 14).

In order to compare the influences of the Bors criteria and the number of hydroxyl groups on the results of the flavonoids in each assay (**Figures 6-13**) are presented. The number of Bors criteria had only a significant influence on the results of the FC assay. The results of the DPPH are not influenced by the number, but by the type of Bors criteria, with the first and the third Bors criterion having the strongest influence. The ABTS and the ORAC assays showed no clear correlation to the number or type of Bors criteria. In contrast to the ABTS assay, the results of the other assays appear to depend on the number of hydroxyl groups, with the values in the ORAC assay decreasing to some extent, if more than five hydroxyl groups are present (11, 14).

3.4. Principal Component Analysis of the DPPH, ABTS, FC and ORAC Results

In order to identify the possible correlations between the assays and analyze structural properties relationship in more detail, a principal component analysis was performed and the results are shown in **Figures 14**, **15**.

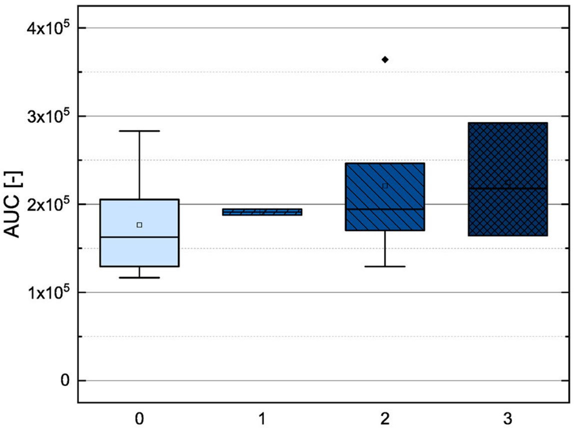

FIGURE 6 | Boxplot of the mean values of results according to their number of fulfilled Bors criteria in the ORAC assay. The range within standard errors (1.5 interquartile range) is represented by error bars.

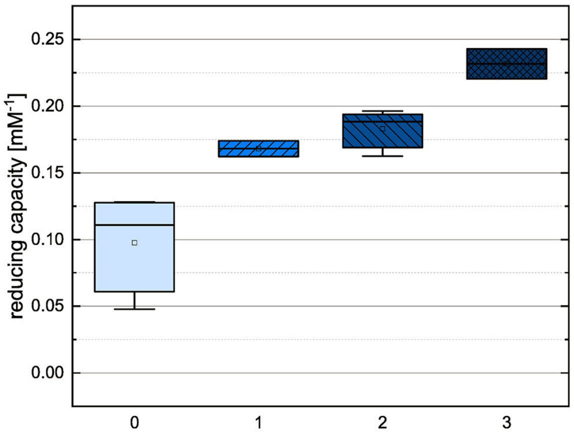

FIGURE 7 | Boxplot of the mean values of results according to their number of fulfilled Bors criteria in the FC assay. The range within standard errors (1.5 interquartile range) is represented by error bars.

Both principal components cover 78.69 % of the initial variance of the original dataset. Principal component 1 represents the antioxidant behavior and depends on the results of all four assays. The higher the loading value of principal component 1 was, the higher the antioxidant effect was considering all four assays. The loading value of the FC assay is mostly correlated with principal component 1. This might be caused by affecting all subgroups equally well and therefore, we conclude that the FC assay is the most suitable to determine the antioxidant effect of the measured substances as previously reported (11). Furthermore, the FC and DPPH assays proceed in opposite direction to the ABTS and ORAC assays regarding principal component 2 and accordingly are influenced by different

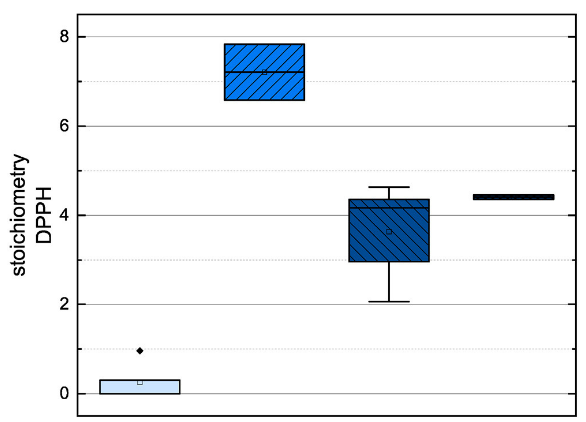

FIGURE 8 | Boxplot of the mean values of results according to their number of fulfilled Bors criteria in the DPPH assay. The range within standard errors (1.5 interquartile range) is represented by error bars.

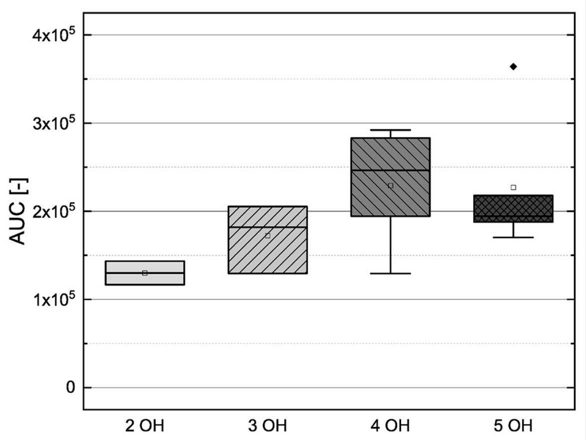

FIGURE 10 | Boxplot of the mean values of results according to their number of hydroxyl groups in the ORAC assay. The range within standard errors (1.5 interquartile range) is represented by error bars.

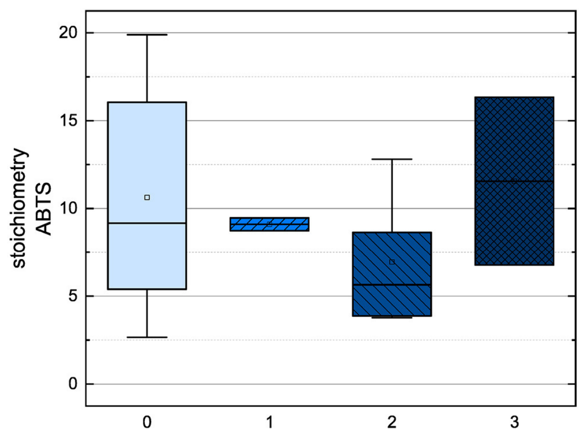

FIGURE 9 | Boxplot of the mean values of results according to their number of fulfilled Bors criteria in the ABTS assay. The range within standard errors (1.5 interquartile range) is represented by error bars.

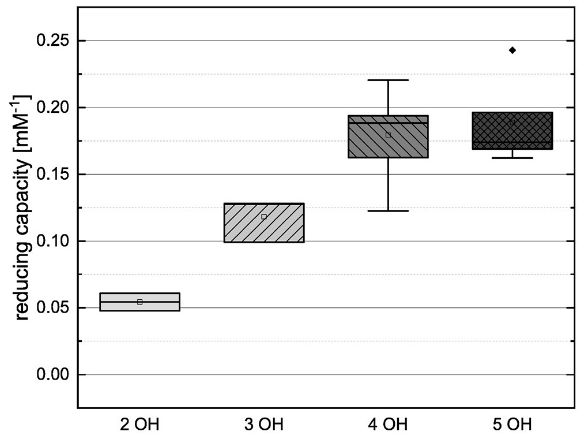

FIGURE 11 | Boxplot of the mean values of results according to their number of hydroxyl groups in the FC assay. The range within standard errors (1.5 interquartile range) is represented by error bars.

structural properties. The loading plots of the FC and DPPH assays point in the same direction, which can be explained as they follow the same reaction mechanism, i.e., SET. Since the loading plots of the ABTS and ORAC assays are very similar, it is possible that they follow the same reaction mechanism in our study. Also in the literature it is often not clear to which reaction mechanisms the ORAC and the ABTS can be assigned, since this depends above all also on the polarity of the solvent used (30, 31, 37, 38, 80, 81).

The score values of the different antioxidants show a clustering according their subgroups in the principal component analysis. Compared with the loading values of the four different assays the ABTS and ORAC seem to be particularly sensitive to the group of dihydrochalcones and rather to detect the substances of the flavanones and flavanols subgroups. For their determination, the FC and DPPH assays seem to be best suited. The substances also arrange with increasing number of hydroxyl groups along principal component 1 and therefore, this structural feature has a strong influence on the antioxidant behavior. With an increasing number of hydroxyl groups, the antioxidant effect increased. The influence of the fulfilled Bors criteria was also analyzed but no clear trend was shown. Nevertheless, an additional Bors criterion could have a positive influence, since for example QUR achieved a higher value than MOR despite having the same number of hydroxyl groups, but different number of Bors criteria.

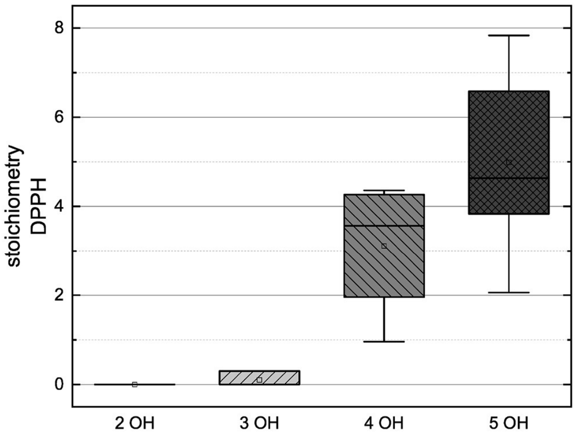

FIGURE 12 | Boxplot of the mean values of results according to their number of hydroxyl groups in the DPPH assay. The range within standard errors (1.5 interquartile range) is represented by error bars.

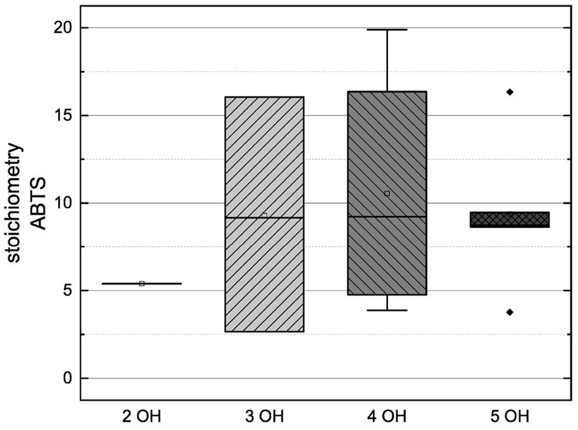

FIGURE 13 | Boxplot of the mean values of results according to their number of hydroxyl groups in the ABTS assay. The range within standard errors (1.5 interquartile range) is represented by error bars.

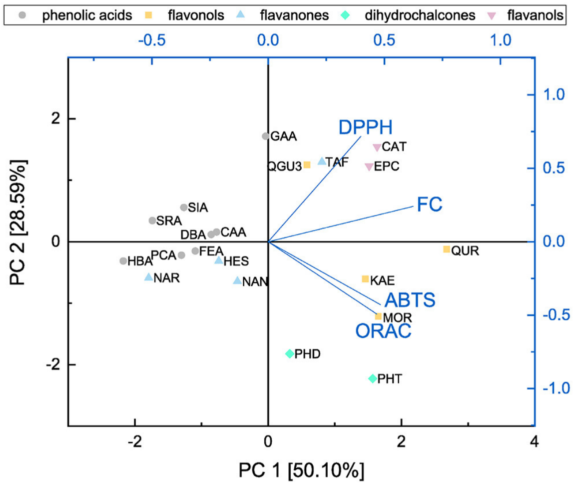

FIGURE 14 | Principal component analysis of results from ABTS, DPPH, FC, and ORAC assays presented as a function of subgroups.

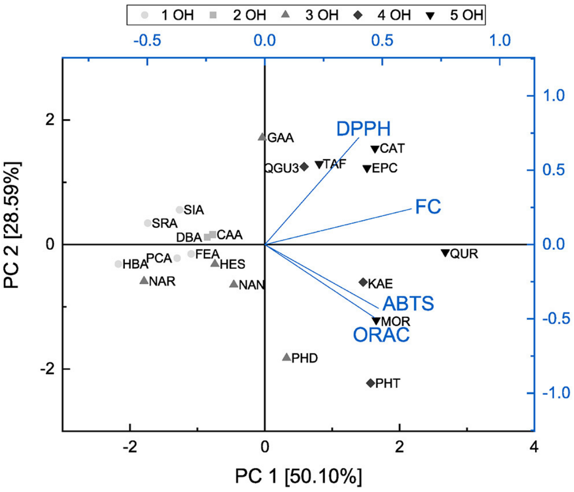

FIGURE 15 | Principal component analysis of results from ABTS, DPPH, FC, and ORAC assays presented as a function of number of hydroxyl groups.

4. CONCLUSIONS

In order to investigate the structural influence of phenolic compounds on the outcome of the ORAC assay, we measured standard references belonging to five different subgroups, namely phenolc acids, flavonols, flavanones, dihydrochalcones, and flavanols. These results were compared to those from the ABTS, DPPH and FC assays, previously published by Platzer et al. (11, 14).

According to literature, the antioxidant behavior of the abovementioned substances is dominated by the substituents, whereas their backbone plays a minor role. This is also true for our results, where the results were similar for subgroups with different backbones. The number of hydroxyl groups present in the substances had the highest influence on the AUC. The more hydroxyl groups, the better the result, except for molecules with two or more hydroxyl groups next to each other. This may be due to steric hindrance. The influence of additional methoxy groups could not be clarified conclusively. A sugar residue at C-7 seems to have no influence on the result, whereas sugar residues at C-3 or C-5 reduced the antioxidant effect. Although, the importance of Bors criteria on the antioxidant behavior is often described in literature, their effect could not be clearly shown in our studies.

Only the second Bors criterion showed a positive effect in the ORAC assay in some cases.

By comparison to the ABTS, DPPH and FC assays, no clear correlation was found and therefore, no general statements can be drawn for all assays. An increasing number of hydroxyl groups had a positive influence on the results of the DPPH, FC and ORAC assays, as long as no steric hinderance occurred. Hydroxyl in *para* position increased the results in the ABTS, DPPH and FC assays, but had a negative influence on the ORAC value. The same applied for a methoxy substitution. In all four assays a sugar residue had either no influence on the results or slightly reduced the antioxidant effect. The three-dimensional structure had no influence on any assay result. The DPPH and FC assays most likely correlated with the Bors criteria, in contrast to the ORAC and ABTS assays, where their effect was not found. Finally, the DPPH was the only assay where some substances did not react at all. The differences in the assays can be explained by the different reaction mechanisms and the use of different evaluation methods, solvents, pH values and model radicals.

Principal component analysis showed the dependence of all four assays on the antioxidant behavior. Furthermore, the FC and DPPH assays depended on different structural properties than the ABTS and ORAC assays and accordingly we assume that they follow different reaction mechanisms. In addition, the number of hydroxyl groups had a strong influence on the antioxidant activity, while an influence of the Bors criteria was only shown partially and in combination with the number of hydroxyl groups.

In summary, our studies revealed the influence of structural properties of substances belonging to the subgroups of phenolic acids, flavonols, flavanones, dihydrochalcones, and flavanols on their antioxidant activities. Little was known about the structural properties of standard references that have an impact on experimental results, especially for ORAC assay. The commonalities of SET- and HAT-based reaction mechanisms were mainly studied theoretically and they were only partly in agreement with our experimental findings. The principal component analysis additionally showed the suitability of the different assays for different subgroups and can be used in further studies to select the appropriate assays for the specific application. For example, the DPPH should be used for flavanol-rich samples, whereas the ABTS and ORAC assays are suitable for dihydrochalcones and non-glycosylated flavonols. The FC assay is the only assay that evaluates all subgroups equally and best represents total antioxidant activity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MP and SK contributed to conception and design of the study. MP, SK, and TT contributed to methodology, data curation, and visualization. MP performed the statistical analysis and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.882458/full#supplementary-material

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4.4 Quantitative structure-property relationship (QSPR) of plant phenolic compounds in rapeseed oil and comparison of antioxidant measurement methods [4]

Summary

Despite the intensive consideration of structure-activity relationships in the antioxidant assays, it has not yet been clarified which assay is best suited for which application. This need exists, for example, in the technical sector such as the lubricant industry, where time-consuming and complex application tests are carried out. Therefore, 20 phenolic compounds (phenolic acids, flavonols, flavanones, dihydrochalcones, and flavanols) were incorporated into rapeseed oil and were measured using rancimat method and P-DSC in order to compare the results with those of four *in vitro* antioxidant assays.

The phenolic components of all subgroups showed an antioxidant effect in rapeseed oil, which is why they were potentially suitable as a natural alternative to conventional antioxidant additives. A high effect was shown here particularly by the Bors criteria. The more of them a substance fulfills, the higher its antioxidant effect. The strongest influence resulted from Bors 1. Statistical methods using PC analysis showed that the results of the DPPH assay correlated best with the oil measurement methods and the assay can therefore be used as a potential alternative to the time-consuming oil measurement methods. This applied for the phenolic acids as well as for the flavonoids. Furthermore, the flavonoids correlated with the FC assay, albeit lower. The *in vitro* assays had some advantages compared to the oil measurement methods, such as shorter measurement time, easy performance, and small sample volume. However, it should be noted that these findings cannot be transferred to the measurement of extracts, since the influence of other ingredients such as sugars, carbohydrates and proteins must be clarified first.

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Author contributions:

- Melanie Platzer: conceptualization, methodology, data curation, visualization, statistical analysis and writing-original draft preparation
- Sandra Kiese: conceptualization, methodology, visualization, writing-review and editing and supervision
- Tobias Asam: methodology, data curation and writing-review and editing
- Fransika Schneider: data curation and writing-review and editing
- Thorsten Tybussek: methodology and writing-review and editing
- Thomas Herfellner: writing-review and editing, supervision and funding acquisition
- Ute Schweiggert-Weisz: writing-review and editing and supervision
- Peter Eisner: writing-review and editing and supervision

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Article

Quantitative Structure-Property Relationship (QSPR) of Plant Phenolic Compounds in Rapeseed Oil and Comparison of Antioxidant Measurement Methods

Melanie Platzer [1,2,*], Sandra Kiese [2], Tobias Asam [3], Franziska Schneider [2], Thorsten Tybussek [2], Thomas Herfellner [2], Ute Schweiggert-Weisz [2,4] and Peter Eisner [1,2,5]

[1] ZIEL-Institute for Food & Health, TUM School of Life Sciences Weihenstephan, Technical University of Munich, Weihenstephaner Berg 1, 85354 Freising, Germany; peter.eisner@ivv.fraunhofer.de
[2] Fraunhofer Institute for Process Engineering and Packaging IVV, Giggenhauser Str. 35, 85354 Freising, Germany; sandra.kiese@ivv.fraunhofer.de (S.K.); franziska.schneider@ivv.fraunhofer.de (F.S.); thorsten.tybussek@ivv.fraunhofer.de (T.T.); thomas.herfellner@ivv.fraunhofer.de (T.H.); ute.weisz@ivv.fraunhofer.de (U.S.-W.)
[3] Carl Bechem GmbH, Weststr. 120, 58089 Hagen, Germany; asam@bechem.de
[4] Institute for Nutritional and Food Sciences, University of Bonn, Meckenheimer Allee 166a, 53113 Bonn, Germany
[5] Faculty of Technology and Engineering, Steinbeis-Hochschule, George-Bähr-Str. 8, 01069 Dresden, Germany
* Correspondence: melanie.platzer@ivv.fraunhofer.de

Abstract: Natural antioxidants are known for their ability to scavenge free radicals and protect oils from oxidation. Our aim was to study the structural properties such as the number of hydroxyl groups and Bors criteria of phenolic substances leading to high antioxidant activity in oil in order to analyze common trends and differences in widespread in vitro antioxidant assays. Therefore, 20 different phenolic substances were incorporated into rapeseed oil and were measured using pressurized differential scanning calorimetry (P-DSC) and the Rancimat method. The Bors criteria had the highest influence on the antioxidant effect in rapeseed oil, which is why myricetin (MYR), fulfilling all Bors criteria, reached the highest result of the flavonoids. In the Rancimat test and P-DSC, MYR obtained an increase in oxidation induction time (OIT) of 231.1 ± 44.6% and 96.8 ± 1.8%, respectively. Due to differences in the measurement parameters, the results of the Rancimat test and P-DSC were only partially in agreement. Furthermore, we compared the results to in vitro assays (ABTS, DPPH, FC and ORAC) in order to evaluate their applicability as alternative rapid methods. These analysis showed the highest correlation of the oil methods with the results of the DPPH assay, which is, therefore, most suitable to predict the antioxidant behavior of oil.

Keywords: oil stability measurements; antioxidant activity; flavonoids; phenolic acids; structure-activity relationship

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1. Introduction

Autoxidation of oils is the decisive factor for their shelf life and results in the formation of free fatty radicals, hydroperoxides and peroxides, which lead to a decreased oil quality and the formation of off-flavors and off-odors [1–5]. Accordingly, the oxidation of oils plays an important role in the food industry, but it also affects the properties of technical oils, such as lubricants [6,7]. Autoxidation can be induced by UV radiation or singlet oxygen and occurs in four steps: chain initiation, chain propagation, chain branching and chain termination [8–10]. It is influenced by the oil's composition and storage conditions, such as free fatty acid content, light penetration and temperature, and can be slowed down or even prevented by the addition of antioxidants [1,11,12].

A common method to increase the oxidation stability of oils is the use of antioxidants. In addition to synthetic antioxidants such as butylhydroxytoluol (BHT) and butylhydroxyanisol (BHA), there are natural substances that are known for their radical scavenging ability [7,13–21]. These can be classified into different subgroups based on their structure, which range from the low molecular weight substances such as phenolic acids to complex components such as flavonoids [22,23]. They are found in numerous fruits and vegetables and are usually obtained by extraction processes using organic solvents [1,24–27]. The antioxidant behavior of several plant extracts and commercially available natural antioxidants, as well as their comparison to synthetic antioxidants, has been studied extensively. Research has focused on the substitution of synthetic substances and investigated their efficacy by comparing different extracts [28–46].

There are several methods to measure the oxidation stability of oils, such as sensory analysis, spectroscopic or fluorescence methods, determination of acid and peroxide values and chromatographic methods [10]. Due to its simplicity, small sample size and automated evaluation, the Rancimat method is particularly popular and is used in various fields from the food industry to biodiesel production [4,47,48]. For the measurement, a sample is heated to a defined temperature. The volatile degradation products formed during oxidation are transferred by an air stream into a measuring cell filled with demineralized water. The device detects the increase in conductivity as a function of time, and the end of the measurement is initiated by the rapid increase in conductivity [4,47,49,50]. This test can be performed at a wide range of temperatures and is therefore suitable for a variety of samples [4,47,49,50].

Another popular method to determine oil stability is measurement by pressurized differential scanning calorimetry (P-DSC), where thermally induced transitions, such as decomposition, melting or the crystallization of samples, which lead to a change in heat capacity, are determined. Due to the increased pressure, the measurement time can be shortened significantly [51–58]. This makes the method suitable for samples that have been mixed with strong antioxidants [58,59]. However, the method is not frequently used because a calorimeter is not standard laboratory equipment [60,61].

In addition, the antioxidant behaviour of natural compounds is often analyzed using in vitro spectrophotometric assays. These are based on different reaction mechanisms and are influenced by various factors such as temperature, measurement duration, and pH. Accordingly, overestimation or underestimation of antioxidant activity may occur [62–64]. Since the extracts are mixtures of different antioxidant substances, the structure-activity relationship (SAR) of the individual substances is not addressed. The in vitro assays are particularly suitable for comparing samples of the same raw material obtained with different extraction parameters and can thus be very useful to evaluate different production steps of food processing [64,65]. However, there is no recognized standard method regarding the measurement and evaluation of these assays, which makes it difficult or even impossible to compare the measurement results with data from the literature [64,66]. Accordingly, these assays are repeatedly strongly criticized, and their use is discouraged [64].

Nevertheless, these assays continue to be used in various fields and, therefore, are still relevant for research studies [64,67–70]. They are popular methods in research and development as well as in the food industry for predicting the antioxidant activity of plant extracts or natural phenolic compounds because they are simple and quick to perform, require only small sample amounts, and simple laboratory equipment is sufficient [62–64,67–75].

Although the in vitro assays are commonly used in literature, and much is known about the reaction mechanisms and the influence of SAR, the correlation of their results with different types of application media, e.g., oils, and which assay should be preferred is still not clarified. Therefore, we determined the antioxidant activity of 20 different phenolic compounds (shown in Table 1) in a lipophilic matrix. After incorporating the antioxidants into rapeseed oil, we analyzed its oxidation induction time (OIT) using the Rancimat method and carried out P-DSC. By comparison of the two methods, the influence of the structural properties of the phenolic compounds on the oxidative stability was studied in more detail. Furthermore, these findings were compared to the SAR of in vitro assays (the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu (FC) and oxygen radical absorbance capacity

(ORAC) assays) published previously [76–78] to experimentally identify a rapid test method suitable to predict the antioxidant activity of phenolic compounds in oil.

Table 1. Phenolic compounds analyzed in this study (phenolic acids and subgroups of flavonoids) along with reference standard names, sample codes and substituents (adapted with permission from Table 1 in [76–78]).

Subgroups	Reference Standards	Code	Position and Substituents						
Phenolic acids			**1**	**3**	**4**	**5**			
	Caffeic acid	CAA	$(CH_2)_2COOH$	OH	OH	H			
	3,4-dihydroxybenzoic acid	DBA	COOH	OH	OH	H			
	Ferulic acid	FEA	$(CH_2)_2COOH$	OCH_3	OH	H			
	Gallic acid	GAA	COOH	OH	OH	OH			
	4-hydroxybencoic acid	HBA	COOH	H	OH	H			
	p-coumaric acid	PCA	$(CH_2)_2COOH$	H	OH	H			
	Sinapic acid	SIA	$(CH_2)_2COOH$	OCH_3	OH	OCH_3			
	Syringic acid	SRA	COOH	OCH_3	OH	OCH_3			
Flavonols			**2′**	**3′**	**4′**	**5′**	**3**	**5**	**7**
	Kaempferol	KAE	H	H	OH	H	OH	OH	OH
	Morin	MOR	OH	H	OH	H	OH	OH	OH
	Myricetin	MYR	H	OH	OH	OH	OH	OH	OH
	Quercetin	QUR	H	OH	OH	H	OH	OH	OH
Flavanones			**3′**	**4′**	**3**	**5**	**7**		
	Hesperetin	HES	OH	OCH_3	H	OH	OH		
	Naringin	NAG	H	OH	H	OH	Rham, Glc		
	Naringenin	NAN	H	OH	H	OH	OH		
	Taxifolin	TAF	OH	OH	OH	OH	OH		
Dihydrochalcones			**4**	**2′**	**4′**	**6′**			
	Phloridzin	PHD	OH	OH	OH	Glc			
	Phloretin	PHT	OH	OH	OH	OH			
Flavanols			**3′**	**4′**	**3**	**4**	**5**	**7**	
	(+)-catechin	CAT	OH	OH	OH	H	OH	OH	
	(−)-epicatechin	EPC	OH	OH	OH	H	OH	OH	

2. Materials and Methods

The following chemicals and reference standards were obtained from Sigma-Aldrich (Steinheim, Germany): caffeic acid (CAA), (+)-catechin (CAT), 3,4-dihydroxybenzoic acid (DBA), (−)-epicatechin (EPC), ferulic acid (FEA), gallic acid (GAA), 4-hydroxybenzoic acid (HBA), hesperetin (HES), kaempferol (KAE), morin (MOR), myricetin (MYR), naringenin (NAN), *p*-coumaric acid (PCA), phloridzin (PHD), phloretin (PHT), quercetin (QUR), sinapic acid (SIA), siringic acid (SRA), taxifolin (TAF), Tween 85 and Span 85. The standard reference naringin (NAG) was obtained from Carl Roth (Karlsruhe, Germany), and copper powder was obtained from Merck (Darmstadt, Germany). Food-grade rapeseed oil from Brökelmann & Co. Oelmühle GmbH & Co. KG (Hamm, Germany) was used. For the experiments, all phenolic substances were dissolved in ethanol absolute.

These solutions were measured in the ABTS, DPPH, FC and ORAC assays. The results were published before and are reproduced here for comparison [76–78]. The measurements were performed according to the literature with some modifications, and detailed instructions can be found elsewhere [76–82].

For incorporation of the phenolic substance ethanol solutions, rapeseed oil was mixed with 0.15 % (w/w) Tween 85 and Span 85 and homogenized for 5 min at 18,000 min^{-1} using a T 25 easy clean control Ultra Turrax disperser from IKA-Werke GmbH & Co. KG (Staufen, Germany). Subsequently, the oil was heated to 50 °C and blended by adding 1510 µL of the phenolic solutions to obtain oils with seven concentrations of polyphenols (0.075, 0.1, 0.25, 0.35, 0.5, 0.75 and 1 mM). The mixtures were first stirred for 2 min and then dispersed for another 2 min using the Ultra Turrax at 16,000 min^{-1}.

For the determination of OIT, 3 g of each oil sample were measured in duplicate together with 300 mg copper powder in a fully automated Rancimat 743 from Metrohm AG (Herisau, Switzerland). A temperature of 60 °C and a gas flow of 20 Lh^{-1} were used. The reaction curves were generated automatically by Rancimat Metrodata version 1.1 software and analyzed using the tangent method [4,47,49,50]. In addition, the oil samples were measured at two concentrations (0.5 and 1 mM) in a P-DSC. These measurements were kindly provided by Carl Bechem GmbH using an ASTM standard (ASTM D6186-19) with some modifications. For the measurements, 1 mg of each sample was subjected to 7 bar and measured at 210 °C. For both measurements, the increase in oxidation stability was calculated compared to the blank (rapeseed oil blended with ethanol, Tween t85 and Span 85).

For both the Rancimat and P-DSC measurements, all prepared solutions of phenolic substances were measured, and the values were calculated for a 1 mM formulation. To compare the results of the OIT measurements and the antioxidant assays, the mean values and standard deviations of 1 mM solutions are shown in Figure 1.

For statistical analysis, one-way analysis of variance (ANOVA) was performed using Sigma Plot (Systat Software, San Jose, CA, USA) based on an unpaired *t*-test. In case of a significant difference, an additional paired test was performed using the Holm–Šidák method. The significance level for both tests was $p = 0.050$, and the statistical analysis was always carried out with all significant decimal places. The statistical analyses were performed for all measured substances. For discussions within phenolic subgroups, the statistical analysis within each group was also performed and referenced at the appropriate points in the text. Multivariate analysis was performed by principal component analysis using OriginPro2018.

3. Results and Discussion

3.1. Analysis of Oil Stability

The increase in OIT of the phenolic compounds in rapeseed oil (calculated for the concentration of 1 mM) using the racimat method and P-DSC are shown in Figure 1.

In both measurement methods, all substances led to an increase in OIT, with the exception of NAG, which showed no effect in the Rancimat measurements. Values in the range of 24.5 ± 4.3% to 231.1 ± 44.6% were obtained for the Rancimat test and 5.5 ± 2.0% to 145.9 ± 16.1% for the P-DSC measurements. Although the results of the two methods were determined by using the same evaluation procedure, different results were obtained, which can be explained by the measurement conditions used. Since the temperature in the P-DSC was much higher than in the Rancimat method, the values obtained were lower. It is known that temperature in particular can have a decisive impact on the stability of phenolic substances and can result in a decrease in antioxidative effect [83]. In some cases, a decrease in antioxidant activity was found even at temperatures of 40 and 60 °C, while in other studies, a decrease was found only at higher temperatures of 100 and 120 °C [83–88]. Thereby, the type of phenolic compounds determines the influence of the temperature. Flavonoids are considered to be particularly temperature-sensitive, and in particular, the subgroups of flavones, flavanols, and flavanones are known to be damaged even at lower temperatures [83,85,89–95]. Accordingly, the numerical values of the two measurement methods are not comparable, and therefore, only the sequences of individual substances will be discussed in the following.

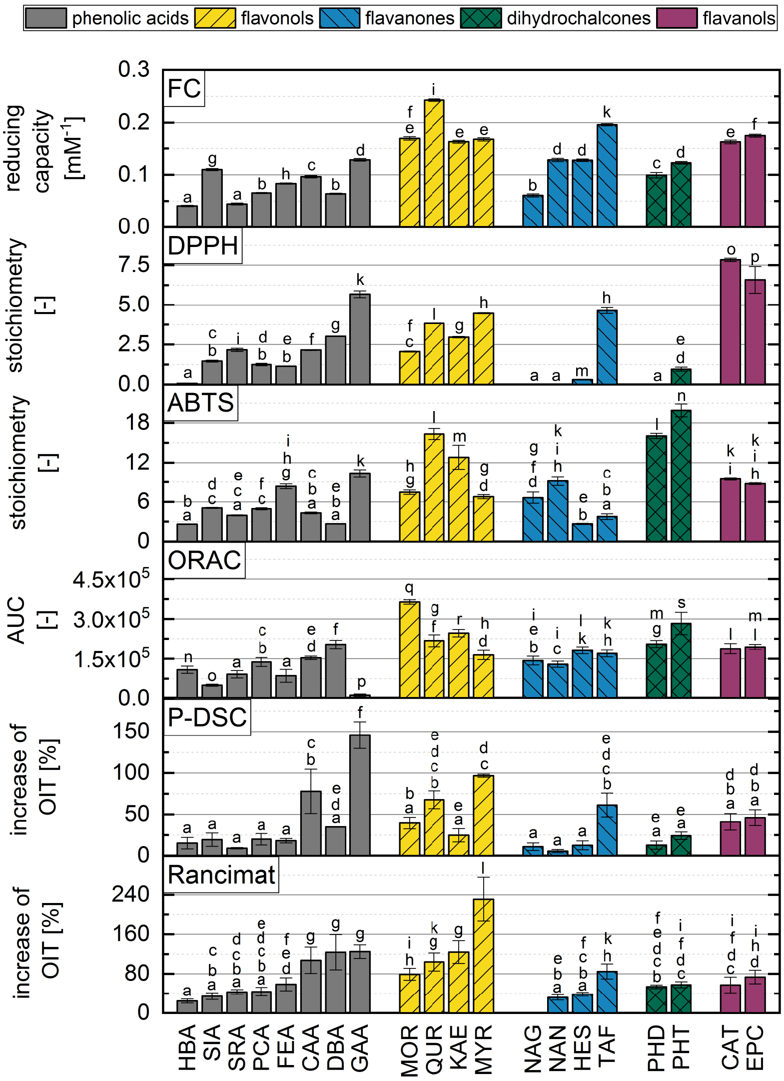

Figure 1. OIT increase in relation to rapeseed oil of all standard reference compounds determined by Rancimat method and P-DSC in comparison to ORAC, ABTS, DPPH and FC assay results (adapted with permission from Platzer et al. [76–78]). The results are shown as mean values with standard deviations for the concentration of 1 mM for all samples and all measured methods. Equal letters indicate that there is no significant difference between the results with an significance level at $p > 0.05$.

In the ranicmat measurements, the highest result was obtained by MYR, which fulfills all three Bors criteria. CAT and EPC, with only Bors 1, obtained lower values than MOR, KAE and TAF, each having two of the three criteria. Thus, for the Rancimat method, the more Bors criteria that were met, the higher the OIT. An exception is QUR, which fulfills all three Bors criteria, but showed a significantly reduced increase in OIT compared to MYR. QUR was more similar to substances with a catechol (CAA, DBA) or galloyl group (GAA), corresponding to Bors 1. Other phenolic acids containing no structural similarity to Bors criteria showed very low values.

In the P-DSC measurements, MYR and QUR showed higher results than KAE, EPC, CAT and MOR. Accordingly, substances fulfilling all Bors criteria seem to be particularly effective. CAT and EPC achieved the same result as KAE and MOR, which suggests that it does not matter whether a substance fulfills only Bors 1 or Bors 2 and 3. The highest result in the P-DSC was obtained by GAA. Phenolic acids with a catechol group also obtained high results (CAA and DBA). It can be concluded that structural properties with high numbers of hydroxyl substituents, such as catechol or galloyl groups, have an influence on the effect in the oil not only for flavanoids.

3.2. Consolidated Analysis of Phenolic Subgroups

In the following section, the sequences in the individual subgroups are discussed, and additionally a significance test is performed within each subgroup.

Considering the phenolic acids, the highest increase in OIT in the Rancimat method was obtained for CAA, DBA and GAA. Since they were not significantly different, it does not seem to make a difference whether a substance has a catechol or a galloyl group. Due to the additional hydroxy group, a higher result for GAA would be expected. However, the opposite effect was already reported in some studies, where the presence of a galloyl group even led to a decrease in antioxidant behavior [96–99]. It is also known from the literature that galloyl groups are used for the formation of superoxide anions with the formation of stable radicals. Accordingly, compounds with this group often have a prooxidant effect, which could explain the outcomes of our study [100]. However, this could also be explained by steric hindrance due to the three substituents on the aromatic ring, as shown previously [76,78]. Moreover, compounds with a catechol group reached a higher increase in OIT than compounds with only one hydroxyl group (DBA and CAA higher than HBA, SIA, SRA, PCA, and FEA). The presence of these groups results in increased stability of the aromatic ring due to electron delocalization leading to high antioxidant activity [101–105]. Furthermore, HBA and SRA did not achieve significantly higher values than PCA and SIA, and it can be concluded that the prolongation of the electron delocalization system also has no influence on the antioxidant effect in oil. It was expected that the hydroxycinnamic acids would reach a higher oxidation stability than the hydroxybenzoic acids due to their additional conjugated double bond. This additional bond leads to an extended electron delocalization and a higher stability of the radical [106–109]. HBA and PCA achieved the same results as SRA, SIA and FEA, so an additional methoxy group also has no influence on the antioxidant effect in oil, which is not in agreement with the literature [98,99] Since these substances, just like GAA, also have three substituents, this result can possibly also be explained by steric hindrance. Methoxy groups are electron donors and thus reduce the dissociation energy, allowing electrons to be released more easily and increasing the antioxidant effect [98,110–112]. However, a lower antioxidant activity was found for FEA compared to CAA, which has a methoxy group instead of a hydroxyl group. This leads to a decrease in active electrons and hydrogen-donating groups and a lower antioxidant activity [106–109,113].

In the P-DSC measurements, the highest value was found for GAA. Due to the presence of a galloyl group, the molecule has the ability to release hydrogen atoms, which is particularly important for hydrogen transfer and is a reason for its high antioxidant activity [98,99]. Since this value was higher than the values of DBA and CAA, an additional hydroxyl group seems to positively influence the increase of OIT. Like the Rancimat results, DBA and CAA reached higher values than HBA and PCA, which only have one hydroxyl group. The more hydroxyl groups a molecule owns, the higher the antioxidant activity, which was also shown before [106,114]. HBA and SIA as well as SRA and PCA did not show significantly different values, which is why an additional methoxy group has also no influence on the antioxidant behavior in oil.

For the flavonols, the highest result in the Rancimat method was obtained for MYR, and therefore, a galloyl group increases the antioxidant effect in this measurement the most, which is in agreement with the literature [115]. A high increase in OIT was expected because the substance meets all of the Bors criteria and, therefore, should achieve the most efficient electron delocalization [116,117]. QUR and KAE did not show significantly different values, and the influence of the catechol group seems to be less strong. Here, a different result was expected, since the delocalization of the B-ring leads to an increased stability of the phenoxyl radical. In addition, the delocalization of the B-ring is further enhanced by the formation of an intramolecular hydrogen bond by the catechol group [102,107,118–121]. The lowest antioxidant effect was obtained for MOR, which is why a negative influence by an additional hydroxyl group at C2′ cannot be excluded (KAE > MOR). This was not expected and is not consistent with the literature [122]. Similar results were obtained for the P-DSC measurements. Since MYR and QUR showed the highest values, the strongest influence seems to be provided by meeting Bors 1. MYR and MOR with 2 and 3 hydroxyl groups reached higher values than KAE with only one hydroxyl group, indicating that the number of hydroxyl groups on the B-ring has a decisive influence on the effect of the substances in oil. In addition to the number, the position of the hydroxyl groups also influences the antioxidant behavior [110,111,123,124]. QUR with one catechol group (corresponding to Bors 1) reached a higher value than MOR, which has one hydroxyl group at C2′ and C4′ of the B-ring.

TAF reached the highest value for the group of flavanones, which is the only substance with Bors 1. An influence of the hydroxyl group on C3 cannot be confirmed by these results, since this property is only fulfilled by TAF. It is therefore possible that, in addition to the catechol group on the B-ring, the C3-OH also has a positive influence on the antioxidant behavior in oil. The higher result of TAF compared to HES was expected, since it is already known from the literature that a hydroxyl group in the *para* position has a positive influence on the antioxidant activity [79,98,99,111,123,125–127]. NAN obtained a higher result than NAG, indicating that the presence of a hydroxyl group at C7 has a positive influence on the antioxidant effect. NAG was the only substance that showed no reaction in the Rancimat method. The reason for this could not be clarified based on our experiments, but it is possible that this effect can be attributed to the sugar residue. In the P-DSC measurements, only TAF reached a significantly higher result than the other substances, again due to Bors 1. NAN, NAG and HES induced the same increase in OIT; therefore, neither the presence of a hydroxyl group at C7 nor an additional methoxy group at C4′ seems to affect the antioxidant activity.

For the dihydrochalcones as well as for the flavanols, both OIT tests showed the same sequences of results. PHD and PHT led to significantly the same increase in OIT, so an additional OH group at C6′ does not affect the antioxidant behavior in the oil. Furthermore, it can be concluded from the results of the flavanols that the structural spatial arrangement also has no influence on an antioxidant effect in the oil. Accordingly, both tests showed the same values for CAT and EPC, which was in agreement with literature [128].

In summary, the results of the P-DSC and the Rancimat measurements show similar trends. Both high and low correlation between these two methods have been reported in the literature [129–135].

3.3. Comparision of Oil Measurement Methods with In Vitro Antioxidant Assays

In order to identify possible rapid test methods to predict the antioxidant effect in oils, the results of the OIT measurements are compared to various in vitro assays, which were previously published [76–78] and are discussed in the following. To simplify comparability, the previously published measurement results of the same phenolic substances from the ABTS, DPPH, FC, and ORAC assays are presented once again, and the results are reproduced from Platzer et al. [76–78] in Figure 1. The in vitro results of MYR were not reported before and were measured for this study using the same measurement and evaluation methods.

For the OIT measurements, a strong influence of the Bors criteria on the antioxidant behavior was found. Furthermore, phenolic substances fulfilling only Bors 1 were similar to substances with Bors 2 and 3. The same behavior was also observed in the FC and DPPH assays, whereas these results were not observed in the ABTS and ORAC assays. In the literature, correlations as well as differences of antioxidant assays with each other and in comparison to oil measurements are reported [62,64,76–78,126,136,137]. In general, it is known that the antioxidant behavior of a substance depends on the position and the number of the hydroxyl groups. Furthermore, it is also known that the nature of the substituents of a molecule influences the antioxidant behavior more than its basic structure [107,109,111,138–142].

For the phenolic acids, the ABTS, FC and ORAC assays showed higher antioxidant activities for substances with a hydroxycinnamic acid group than for substances with a hydroxybenzoic acid group, which is not in agreement with the results of the DPPH assay and the oil measurements. As already known from the literature, the number of hydroxyl groups has an influence on the antioxidant behavior. This was confirmed in the DPPH and FC assays, as well as in the OIT measurements. The more hydroxyl groups a substance has, the higher the antioxidant effect. These results were not shown for the ORAC and the ABTS assays. Here, substances with one galloyl group sometimes even achieved lower values, which may be due to steric hindrance. The influence of an additional methoxy group also affects the methods differently. While this has no influence on the antioxidant behavior in the ABTS, DPPH, FC assays as well as in the OIT measurements, it even positively influenced the results obtained in the ORAC and ABTS assays.

Additionally, for the group of flavonols, different results were found. While the presence of Bors criteria does not play a role for the results in the ABTS and ORAC assay, the influence on the remaining assay seems to be even higher. Both the number and the type of Bors criteria is crucial here. In the DPPH and FC assays as well as in the OIT measurements, the highest influence was shown by Bors 1. Thus, substances with a catechol group on the B-ring achieved higher antioxidant activities than substances with only an hydroxyl group. In the DPPH assay and in the P-DSC measurements, substances with a galloyl group also showed the highest activity. The fact that this result cannot be transferred to the other test methods may be due to steric hindrance.

In all measurement methods, a positive influence of Bors 2 and 3 on the antioxidant effect was found for the flavanones. While a positive effect of a hydroxyl group in the para position was also found for all in vitro tests, this result could not be conclusively confirmed in the oil measurements, but a positive influence is probable. The presence of a sugar residue at C7 showed a slightly negative influence in all methods or did not affect the antioxidant activity. Furthermore, an additional methoxy group showed no effect on the antioxidative behavior in any method, with the exception of the ORAC assay. In the DPPH, FC and ORAC assays as well as in the two OIT measurements, the highest influence was observed for Bors 1.

In all in vitro methods, a higher antioxidative behavior was found for a substance with an additional hydroxyl group at C6′. This was not the case for the two OIT measurements. Here, no significantly different results were obtained, which means that a positive effect of the hydroxyl group, just like a negative one of the sugar residue, can be excluded. In all methods, a different three-dimensional arrangement of two structural isomers does not seem to influence the result. The two flavanols gave similar activities in all tests.

3.4. Principal Component Analysis of the DPPH, ABTS, FC, and ORAC Assays and OIT Measurements

To better identify the correlations and differences of the in vitro and OIT measurements, principal component analysis was performed. First, a principal component (PC) analysis was carried out on all measured substances, and the results were distinguished with respect to the number of hydroxyl groups (Figure 2) and their different structural properties together with the respective subgroup (Figure 3).

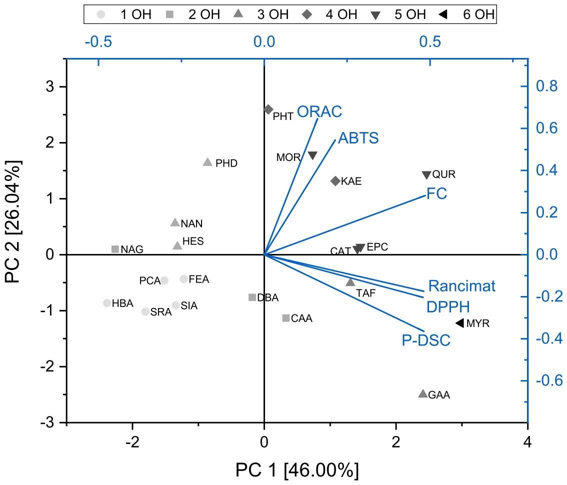

Figure 2. PC analysis of results from Rancimat and the P-DSC, ABTS, DPPH, FC, and ORAC assays presented in dependence of number of hydroxyl groups.

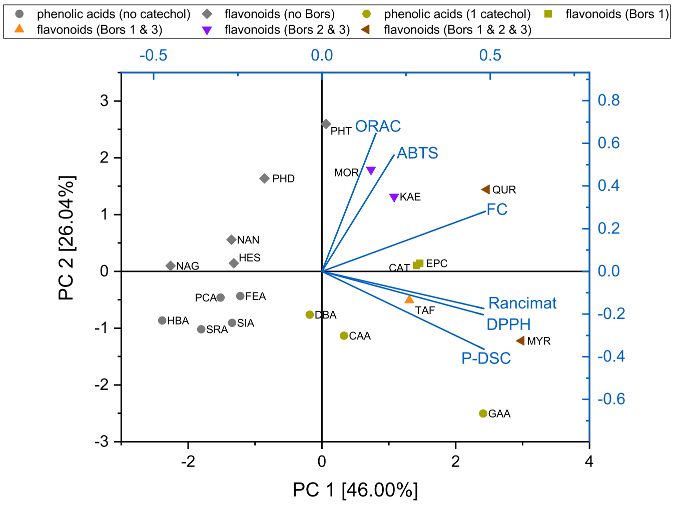

Figure 3. PC analysis of results from Rancimat and the P-DSC, ABTS, DPPH, FC, and ORAC assays presented in dependence of subgroups and structural properties.

Both PCs cover 72.04% of the initial variance of the original dataset. For PC1, both OIT and all in vitro measurements showed positive values. Therefore, PC1 corresponds to the total antioxidant effect, and a high score value in PC1 indicates a high activity. Both OIT measurements showed high positive loading values of 0.48 for PC1. This means that they strongly correlated to the total antioxidant effect. Additionally, the FC (0.49) and DPPH assays (0.48) showed high positive loading values for PC1. Therefore, these measurements are very suitable for the determination of the total antioxidant activity. PC2 showed a separation of the different methods. The ORAC, ABTS and FC assays showed positive loading values of 0.65, 0.55 and 0.28, respectively. In contrast, the Rancimat, DPPH and P-DSC measurements showed negative loading values of −0.17, −0.20 and −0.37, respectively. This indicates that the different methods may be based on different reaction mechanisms. Under this assumption, the ORAC, the ABTS and the FC assays possibly react according to a similar reaction mechamism. However, it should be noted that the loading plots of the ORAC and ABTS shows a higher correlation with each other than with the FC assay. Furthermore, the results show that the loading plots of the OIT measurements correlate well with that of the DPPH assay. Thus, the DPPH assay is the appropriate rapid test to predict the effect of antioxidants in the oil. Such a correlation has also been reported in the literature for the measurement of nut oils [143].

Figure 2 also shows the influence of the hydroxyl groups on the antioxidant effect. Substances with a low number of hydroxyl groups showed lower values for PC1 and PC2 than substances with a high number, which means that the number of hydroxyl groups affects the values of all methods. Considering this observation, the ORAC, ABTS and FC assays showed a higher correlation to the number of hydroxyl groups than the other measurements. However, there are also some exceptions, such as GAA, which indicates that there are other influencing factors. These are shown in Figure 3, where the PC analysis is presented in dependence of subgroups and different structural properties. Here, it can be shown that substances without a catechol group and/or none of the Bors criteria also have the lowest antioxidant effect. Substances with a catechol group, on the other hand, have a higher antioxidant effect. It can also be concluded that substances fulfilling the first Bors criterion show a similar antioxidant effect than substances fulfilling Bors 2 and 3. Thus, Bors 1 seems to have the strongest influence on the antioxidant behavior for phenolic compounds. Since the results for substances fulfilling Bors 1 and 3 show a similar antioxidant effect than the substances fulfilling only Bors 1, the influence of Bors 3 criterion seems to be rather small. The highest PC1 value and thus also the strongest antioxidant effect was shown by substances fulfilling all three Bors criteria. Additionally, the influence of a galloyl group also has a strong effect on the antioxidant behavior in most methods, which is why GAA also achieved a high value, as well as MYR.

To discuss the structural differences and similarities between flavonoids and phenolic acids, PC analysis was repeated for these two groups (Figures 4 and 5). The FC and DPPH assays showed very similar loading values as the Rancimat and P-DSC measurement, which means that these four measurements lead to similar results for prediction of the antioxidant activity of flavanoids. Both the ABTS and the ORAC assay are neither suitable for measuring the antioxidant effect of flavonoids nor for predicting their effect in oil. Just as in the other PC analysis, substances that fulfill none of the Bors criteria show the lowest antioxidant activity, and substances that fulfill all three Bors criteria show the highest activity. In addition, Bors 1 seems to have the highest influence, since substances achieved similar values to substances fulfilling Bors 1 and 3 or Bors 2 and 3. It can also be concluded that the influence of Bors 3 is rather low.

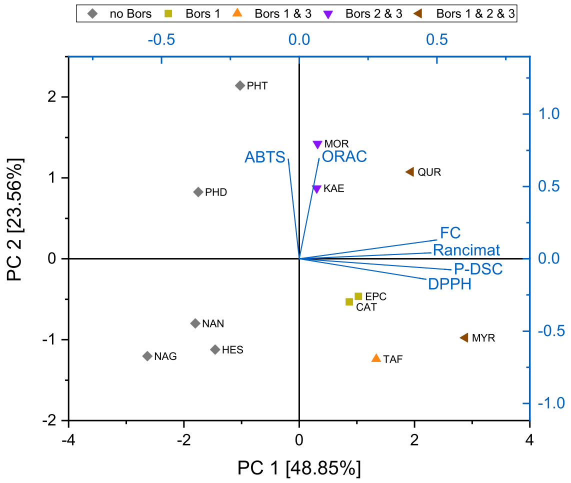

Figure 4. PC analysis of results of flavonoids from Rancimat and the P-DSC, ABTS, DPPH, FC, and ORAC assays presented in dependence of Bors criteria.

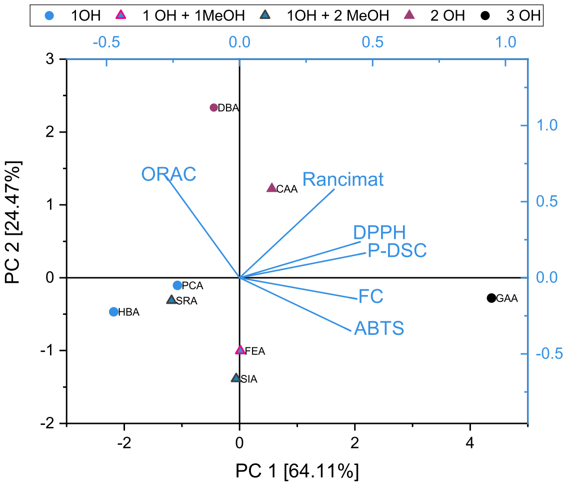

Figure 5. PC analysis of results of phenolic acids from Rancimat and the P-DSC, ABTS, DPPH, FC, and ORAC assays presented in dependence of hydroxyl and methoxy groups.

Figure 5 shows the results of the PC analysis for the phenolic acids. This analysis differs strongly from the analysis shown above. Both OIT measurements, Rancimat and P-DSC, showed similar but slightly different loading plots. Thus means, that for measurement of the antioxidant effect of polyphenols, both measurements will lead to different results. The DPPH assay shows loading values in between the Rancimat and P-DSC method. Therefore, the DPPH assay is the most suitable assay to predict the antioxidant effect of a phenolic acid in oil. Additionally, it can be concluded that the ORAC is less suitable to determine the antioxidant effect of phenolic acids. Considering the results of the individual substances, the values seem to depend on the number of substituents. The lowest results were obtained for substances with only one hydroxyl group and the highest for GAA, which has three hydroxyl groups. From the results, it is clear that additional methoxy groups also have a positive influence on the antioxidant behavior of the phenolic acids.

4. Conclusions

In our study, it was shown that especially the Bors criteria influence the antioxidant behavior of phenolic substances in oil. Basically, the more Bors criteria are fulfilled, the higher the antioxidant effect, with the first Bors criterion having the strongest influence. It was also shown that the results of the Rancimat method are partially in agreement with those from P-DSC. Differences in the two methods can be justified by different temperatures and other measurement parameters.

By performing different PC analyses, the antioxidant effects of phenolic substances in oil were compared with the antioxidant behavior in different in vitro antioxidant assays. There was an appropriate correlation of the OIT data with the results of the DPPH assay. Thus, this represents a good alternative to the oil methods and is suitable for predicting the antioxidant behavior in oil. This is a great advantage since the DPPH assay is a rapid method that can be performed in any conventional laboratory without complicated analytical equipment. In addition to cost and time savings, this also reduces the amount of sample required. Another advantage of the DPPH assay is that no complex sample preparation is required. Additionally, the FC showed a high correlation with the OIT values, so this assay could also be an alternative method. However, when PC analysis is performed only for the phenolic acid subgroup, different results are shown. Here, the OIT measurements in addition to the DPPH and FC assay also correlate with the antioxidant activity of the ABTS assay, but the correlation between the assays is not as clear as in the other PC analysis.

In summary, the in vitro antioxidant assays are suitable for predicting the antioxidant activity of polyphenolic standard references in oil. In our results, the highest correlations for the different phenolic subgroups were found for the DPPH assay. However, to use the method for the characterisation of natural extracts, further influences, e.g. pH, sugar and protein content, on the correlation have to be assessed first.

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Abbreviations

ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
BHT	butylhydroxytoluol
BHA	butylhydroxyanisol
CAA	caffeic acid
CAT	(+)-catechin
DBA	3,4-dihydroxybenzoic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EPC	(−)-epicatechin
FC	Folin–Ciocalteu
FEA	ferulic acid
GAA	gallic acid
HBA	4-hydroxybenzoic acid
HES	hesperetin
KAE	kaempferol
MOR	morin
MYR	myricetin
NAG	naringin
NAN	naringenin
OIT	oxidation induction time
ORAC	oxygen radical absorbance capacity
P-DSC	pressurized differential scanning calorimetry
PC	principal component
PCA	*p*-coumaric acid
PHD	phloridzin
PHT	phloretin
QUR	quercetin
SIA	sinapic acid
SAR	structure-activity relationship
SRA	siringic acid
TAF	taxifolin

5 General Discussion, Conclusion and Outlook

Due to the increasing health and enviromental awareness of the population, the demand for natural antioxidants for the lubricating industry has continued to grow in recent years. Therefore, many companies try to replace harmful substances by natural antioxidants, which can be found in a variety of plants, fruits and vegetables [3–7]. The most widespread of these substances are polyphenols, which are known for their ability to scavenge reactive oxygen species and other free radicals and therefore have a high antioxidant effect [23–29]. This activity depends on different structural properties, i.e., the overall structure as well as the type and position of side groups [130, 173]. A well-known classification of the structurally decisive properties for the antioxidant effect are the Bors criteria, which were developed especially for the group of flavonoids [174, 248]. Further important properties are the number and position of the hydroxyl groups or other sidegroups like sugar residues or methoxy groups on the molecule [176–184].

A series of *in vitro* assays, e.g., the ABTS, DPPH, FC, and ORAC, was developed for using model radicals to determine the antioxidant activity of the molecules. These assays are often criticized due to many drawbacks such as poor comparability with each other, or non-standardized measurement and evaluation methods [218, 234]. Although the assays are frequently used in the literature, there were some knowledge gaps regarding the structure-activity relationships that affects the outcome of each assay. It was also unknown, which assay was suitable for measuring which type of polyphenol and their correlation to application-based test methods. The assays, despite their reputation, still are frequently used as standard laboratory analysis. This is mainly due to the fact that they are simple, cheap and quick to perform and do not require any special training or laboratory equipment.

So far, the assays have been used particularly in the food industry and not in technical areas such as the lubricant industry [218, 249]. Here, application-based tests such as the rancimat method or P-DSC are among the common standard analysis [80, 90, 91]. Since these tests cannot be performed in every standardized laboratory and are both complex and time-consuming procedures, the implementation of the *in vitro* antioxidant assays would add great value. Therefore, further intensive studies were needed to consider the mechanisms behind the different assays and the application-based methods to evaluate which structural features lead to high antioxidant activity. Furthermore, there was a need to clarify which assay correlates with the antioxidant effect of the polyphenols in the oil application tests.

The differences and the similarities of *in vitro* assays are first discussed in the following Section 5.1 to clarify these open points. For this purpose, the influence of structural properties on the results is described in more detail. The influence of the number and position of the hydroxyl groups (Section 5.3) as well as the influence of other substituents such as sugar residues, methoxy, and acid groups (Section 5.4), is elaborated in addition to the Bors criteria (Section 5.2). The same structural properties are also discussed for the application-based oil methods, the rancimat method and the P-DSC, and the results are compared (Section 5.5). Finally, also reasons will be given whether phenolic substances are suitable for use in technical oils such as lubricants and if further research is needed (Section 5.6).

5.1 Similarities and differences of in vitro antioxidant assays

The fact that antioxidant activity measurement methods are based on two different reaction mechanisms, i.e., SET and HAT, was known in the literature long before this thesis [185]. Both mechanisms always occur in parallel and which of the two is dominant depends on factors such as the type of radical and the type of solvent [186–189]. Furthermore, there are different measurement and evaluation methods, which makes it difficult to draw conclusions about the individual structural properties of each assay [218, 234]. Accordingly, whether there is a correlation between SET- and HAT-based assays was discussed contradictorily in the literature, and further research was needed [206, 218, 234–237].

For a better comparability of the results, the same concentrations of phenolic substances and, if possible, the same methods for measurement and evaluation were used within this thesis. This was possible in the case of the ABTS and DPPH assay. It became apparent that phenolic substances can have different reaction rates and sometimes require several hours to reach the end point of the reaction, which can result in underestimation if the measurement period is too short. By adopting the second-order kinetics, it was possible to determine the endpoint of the reaction without underestimating the antioxidant effect even with a shortened measurement duration of 30 min. Since in the literature often a fixed measurement duration is used without recording kinetics, a literature comparison is only partially possible due to low comparability and different results [196, 198, 199].

Despite the adjustment of the measurement method and the use of the same concentrations of phenolic substances (phenolic acids, flavonols, flavanones, dihydrochalcones and flavanols) no correlation was found when comparing the results of the SET- (DPPH, ABTS, FC) and HAT-based (ORAC) assays. The assays depended on different structural properties, such as the Bors criteria, the number of hydroxyl groups, and the presence of other substituents, and did not present the same trends. The only commonality was, that compounds that met three Bors criteria achieved the highest antioxidant activity in the ABTS, DPPH, and FC assay.

In addition, it was possible to identify the structural properties that cause a high value in the FC assay. Five classes were established, which allow the classification of phenolic substances in terms of their antioxidant activity, i.e.:

- class 1: 2 OH groups and none of the Bors criteria
- class 2: 3 to 4 OH groups and none of the Bors criteria
- class 3: one of the Bors criteria
- class 4: two of the Bors criteria
- class 5: all of the Bors criteria

With this new classification, it is possible to predict the antioxidant effect in the FC assay based on the chemical structure.

Furthermore, substances of the subgroups of flavanones and dihydrochalcones did not react with the DPPH radical. Therefore, one of the other assays should be preferred if samples rich in flavanones or dihydrochalcones or samples with unknown compositions are to be measured.

There are also different results in the literature about the correlation of the assays, regardless of whether they react according to the same reaction mechanism or differently. These involve both experimental and theoretical studies [187, 189, 190, 193, 206, 216–218]. However, it should be noted that influencing factors such as the type of solvent, the pH, and others are not always taken into account in the theoretical studies. In addition, in many *in vitro* assays, model radicals are used, which are intended to simulate oxygen radicals or fatty acid radicals from the application-based tests. However, steric hindrance of the molecules may occur here, which is why the results do not necessarily correlate. This does not apply to the FC assay, since no model radicals are used here and the reaction is based on a reduction of the FC reagent.

Although the comparison of the four *in vitro* assays initially showed only low agreement, it was possible to identify similarities and differences by performing a PC analysis. Each of the assays indicated antioxidant activity and thus is suitable to determine it, with FC showing the highest level of agreement. Furthermore, the antioxidant activity depended on both the number of hydroxyl groups and the number and type of Bors criteria. As the number of hydroxyl groups increased, so did the results in the assays, whereas there was no clear trend for the Bors criteria. It was also found that the ABTS and ORAC assays are particularly suitable for measuring dihydrochalcone-rich samples, whereas the FC and DPPH assays should be preferred for samples rich in flavanones and flavanols. In general, a correlation of the ABTS and ORAC, and the FC and DPPH assay was found, which could indicate of the same reaction mechanism. It is known from the literature that the FC and DPPH follow the SET, which matches the results of this thesis [185, 250]. The classification of the ABTS and ORAC assay in the literature, on the other hand, is not always clear. Therefore, they possibly could follow the same reaction mechanism, even if the assays were originally assigned to different mechanisms [251–256]. To better understand the structure-activity relationship behind the assays and to learn more about the structural properties, these will be discussed in more detail below.

5.2 Influence of the Bors criteria on the in vitro assays

A well-known classification of the structure-activity relationships, which is responsible for a strong antioxidant effect, are the Bors criteria [174, 248]. However, whether the different *in vitro* assays all depend equally on the Bors criteria and whether their type or number is responsible for a high effect was rarely reported and therefore further research was needed.

In this thesis the Bors criteria had a different influence on the four assays. For the ABTS and ORAC assay, neither the number nor the type of Bors criteria had a positiv effect on the antioxidant activity. In the ABTS assay, there was to some extent a positive influence on the subgroup of flavonols. Thus, the results were only partially consistent with data from the literature [174, 223, 248]. The strongest influence of the Bors criteria was shown for the FC assay. Here, both the number and the type of Bors criteria had an impact on the result. Since it was already known from the literature that both the type and the number of Bors criteria have an influence on the antioxidant effect, this result was consistent [174, 248]. As described before, no model radicals are used in the FC, which may be a possible explanation for the different dependencies.

Although the number of Bors criteria played a decisive role only in the FC assay, the phenolic compound quercetin scored highest in the ABTS, DPPH, and FC assay for the subgroup of flavonols. This result was expected since substances fulfilling all three Bors criteria are reported to have high antioxidant activity [174, 248]. However, this result was not shown for the ORAC assay. Myricetin, which also fulfills all three Bors criteria, behaved differently in the assays. While the compound in the DPPH assay achieved the highest value for the flavonols, this did not apply to the other assays. Possibly, due to the galloyl group, there is a steric hindrance in the reaction with the model radical and thus an underestimation of the result. It is already known from the literature that the presence of a galloyl group can influence the antioxidant effect both positively and negatively [257, 258]. It could not be clarified on the basis of these results why the FC assay also gave a low value.

Even though the influence of the Bors criteria was different in each of the assays, the strongest influence was shown for **Bors 1**, which is consistent with the literature [174, 223, 248]. This effect was particularly evident in the DPPH and FC assay and could be applied to all subgroups, with the strongest effect for the flavonols subgroup. In the ABTS assay, however, this positive effect could be shown only partially and not for all subgroups. In the HAT-based ORAC assay, just like the ABTS assay, there was no clear trend regarding the Bors criteria, but it seemed that Bors 1 partially influences the results. A positive effect of Bors 1 was expected and can be explained by the increased stability of the antioxidant radical formed, resulting in a high antioxidant effect [248]. A galloyl group on the B-ring, according to the extended definition, also is attributed to the first Bors criterion. However, substances fulfilling this properties only achieved high values in the DPPH assay, which can again be explained by steric hindrance.

Just like Bors 1, **Bors 2** showed a positive influence on all assays. These observations were only valid for the subgroup of flavonols, because all other groups did not fulfill Bors 2 due to their basic structure. While there was only a slight influence on the DPPH and ORAC assay, Bors 2 was more evident in the

ABTS and FC assay. The reason for the positive influence of Bors 2 was due to increased electron delocalization and was already shown in the literature [174, 248].

Also **Bors 3** showed a positive effect for all assays. For the DPPH assay, this was particularly evident for the group of flavanones, but also for flavonols. The same applied to the FC assay. In the ABTS assay, on the other hand, only the flavonols showed this effect. In the ORAC assay, Bors 3 had only a very small influence. The positive influence on the antioxidant activity of Bors 3 was already shown in the literature and can be explained by the electron delocalization via hydrogen bonds [174, 248].

Based on the results, the influence of the Bors criteria of each assay was not obvious. It was shown that the assays are partly influenced by the type, but also by the number of Bors criteria fulfilled. However, a correlation between the reaction mechanisms and the Bors criteria could not be found. Furthermore, other structural properties were not included in the Bors criteria and, therefore, further research was needed to elucidate the structure-activity relationships of the individual assays in detail. It should also be noted that the Bors criteria can only be applied to the flavonoid subgroup and not to phenolic acids. Accordingly the number of hydroxyl groups and other structural properties should also be considered.

5.3 Influence of the number and position of hydroxyl groups on the in vitro assays

In this thesis, the number of hydroxyl groups had a positive effect in all assays, with the lowest influence in the ABTS and the strongest in the FC assay. For the ORAC assay, it was also found that the number of hydroxyl groups influences the activity, especially if more than five. This influence was particularly strong in all assays for the subgroup of phenolic acids. Here, a correlation between the number of hydroxyl groups and the antioxidant potential occurred, which agrees with the literature [259, 260]. However, it should be noted that not only the number but also the arrangement of the hydroxyl groups can influence the antioxidant effect [223, 261, 262]. For example, the stability of the B-ring is increased by the presence of a catechol group compared to a hydroxyl group, and this increases the antioxidant effect [263–267]. In order to discuss the positions of the hydroxyl groups in a dated and systematic way, they are subsequently described below on the basis of their position on the rings.

The phenolic substances used in this thesis had hydroxyl groups at position C5 and C7 of the **A-ring**. Since all substances used had a 5-OH group, only the influence of the 7-OH group will be discussed below. This groups showed a positive influence in the FC assay when comparing quercetin-7-D-glucoside and quercetin. It should be noted, however, that in quercetin-7-D-glucoside the hydroxyl group has been replaced by an electron donating group, a sugar residue, why the reduction capacity is reduced and the antioxidant effect deteriorating [268]. Since quercetin-7-D-glucoside was determined only in the FC assay, no statement was made about the other assays. However, it was assumed that a positive effect also would be evident there.

The influence of the hydroxyl groups on the **B-ring** was highest regarding the antioxidant activity, which was in agreement with the literature [254, 264, 269–272]. The presence of a catechol group compared to a hydroxyl group led to higher antioxidant activity in the FC and DPPH, but only partially in the ABTS and ORAC assay. This fact partially agrees with the literature [259, 260]. The presence of a catechol group results in increased electron delocalization across the aromatic ring and thus to increased stability [263–267]. If a phenolic compound had only one hydroxyl group on the B-ring, the position was crucial for the antioxidant effect. In the ABTS, DPPH and FC assay, substances with a hydroxyl group in *para* position had the highest values, whereas hydroxyl groups in *meta* and *ortho* position tended to have a lower influence. The ORAC assay did not show this observation. A hydroxyl group in *para* position has a stronger effect on the antioxidant effect and increases it by stabilizing the phenoxyl radical through intramolecular hydrogen bonds [196, 217, 225, 261, 262, 273–275].

Furthermore, a molecule with a **galloyl group** on the B-ring reached a higher antioxidant activity in the DPPH assay due to increased electron stability of the aromatic ring [225, 273]. For the ABTS and FC assays, a positive effect was shown for the phenolic acid subgroup, but not for the flavonoids, whereas a galloyl group in the ORAC assay even led to a reduction of the antioxidant effect for all subgroups, which was already reported in the literature [225, 257, 258, 273]. This could also be caused by steric hindrance between the phenolic compound and the radical.

If a molecule had an additional **hydroxyl group at C-2'**, an intramolecular hydrogen bond with the 3-OH on the C-ring lead to a stabilization of the O-radical and thus increased the antioxidant effect in the FC and ORAC assay [260, 276]. The other *in vitro* assays did not show this effect, which only partially agrees with the literature [277].

The influence of a 4-oxo group without the 2,3 double bond could not be clarified in this thesis, since all phenolic substances used had this group. Therefore, there was no further discussion of the influence of this group.

In summary, the assays only partially correlated with each other, even after a closer look at the position of the hydroxyl groups. One common feature, however, was the strong influence of the hydroxyl groups on the B-ring. Since it was already known from the literature that other substituents besides the hydroxyl groups, such as sugar residues, methoxy, and acid groups, affect the antioxidant activity, these were investigated further.

5.4 Influence of substituents on the in vitro assays

A slightly negative or no influence of the **sugar residue** on the antioxidant effect was found in all assays. A negative influence could only be shown for the group of dihydrochalcones, but it should be noted that the effect can also be caused by the missing hydroxyl group. In the ORAC assay, a lower value was obtained for quercetin-3-D-glucoside than for quercetin. Based on our data, it could not clarify whether the sugar had a negative and prooxidative effect on the result or whether the missing hydroxyl group caused the drop in antioxidant activity. However, since quercetin-3-D-glucoside did not fulfill Bors 3 due to the missing 3-OH group, it was assumed that the lower value can be attributed to this effect, since the reaction with the free radical is supported by this group and the electron donor effect is favored [278, 279]. Both Bors 2 and a 3-OH group additionally influence electron delocalization and thus increase antioxidant activity [269–272].

Although it was known from the literature that **methoxy groups** can influence the antioxidant behavior, this effect was only found for the ORAC assay for the group of flavonoids [225, 273]. Here, a negative influence on the result was found, if a molecule had an additional methoxy group, which was consistent with the literature [269, 280–284]. In contrast, in the FC a positive influence was shown. For the subgroup of phenolic acids, the value in the DPPH assay was positively affected by the presence of an additional methoxy group, which was consistent with the literature [225, 273]. The same result was obtained for the ABTS and FC measurements. Since methoxy groups are electron donors, the dissociation energy of the bond is decreased, electron transfer is facilitated, and antioxidant activity is increased [285].

Furthermore, a higher value for the antioxidative potential of hydroxycinnamic compared to hydroxybenzoic acid was obtained for the ABTS, DPPH, and FC assay. It was already known from literature that **hydroxycinnamic acids** have a higher antioxidant potential due to their additional conjugated double bond and the resulting extended electron delocalization [260, 269, 286, 287]. This effect was not demonstrated for the ORAC assay, and further studies were needed to clarify the reason for this.

In addition, this thesis demonstrated that the three-dimensional arrangement of phenolic compounds had no effect on the antioxidant activity of the four assays. Other studies also showed this result [288]. Thus, due to the changed structural arrangement, there seemed to be no impairment of the reaction by steric hindrance of the molecules.

Based on the individual structural features, it was possible to identify the crucial structural properties of each assay. If *in vitro* assays should be used to predict the results of an application-based test or not these findings played an important role. However, to use such predictions, it was first necessary to perform the application-based assays with the same phenolic compounds to explore the structure-activity relationships in each application. For oil-based and application-based measurement methods, the rancimat method and the P-DSC, these measurements were performed within this thesis and the results are discussed in Section 5.5.

5.5 Structure-activity relationshisp of phenolic compounds in rapeseed oil

Although both, rancimat method and P-DSC, are standard methods and are often dicussed in the literature, not much is known about the structure-activity relationships of phenolic standard references [43]. Therefore, further research was needed to explore the structurally important properties responsible for high antioxidant activity in oil. For this purpose, 20 different phenolic substance ethanolic solutions were incorporated by using Tween and Span solubilizer, and the influence of Bors criteria, the number and position of hydroxyl, methoxy, and acid groups, and the three-dimensional arrangement was investigated.

Just as in the *in vitro* assays, the **Bors criteria** seemed to be decisively important for the antioxidant activity in rapeseed oil. Especially their number had a significant effect. If all of the Bors criteria are met, the electronscan delocalize best within the molecule, resulting in a high antioxidant activity [248, 289, 290]. While the influence of Bors 1 was weak for the flavonols, this criterion seemed to be particularly important for the flavonols. Bors 2, on the other hand, had no effect on the result in the P-DSC but a slightly positive one for the rancimat method. The influence of Bors 3 could not be clarified within this thesis, but it was assumed that the influence is rather small.

Also, a high **number of hydroxyl groups** lead to a high antioxidant effect in oil. Especially in the group of phenolic acids, the number seemed to be decisive, particularly for the subgroup of flavanones and dihydrochalcones. In addition to the number, the **arrangement of the hydroxyl groups** also played a role here. Thus, substances with one catechol group, i.e., substances possessing only one hydroxyl group or two separately arranged hydroxyl groups, mostly showed a higher antioxidant effect. This effect was explained by the increased electron delocalization and the associated increased stability of the phenoxyl radical, affecting antioxidant activity. The formation of an intramolecular hydrogen bond can further enhance the delocalization of the B-ring [254, 264, 269–272]. From the results of this thesis, it could not be excluded that the presence of a C2'-OH group negatively affected antioxidant activity. However, it was assumed that the lower result of morin compared to quercetin was attributed to the missing catechol group since a negative influence of C2'-OH was not described in the literature [277]. While flavanols with a galloyl group reached higher values in both methods, the result differed in the phenolic acids group. In the P-DSC measurements, substances with a galloyl group were found to have higher antioxidant activity than groups with a catechol group. By the rancimat method, on the other hand, similar results were obtained for the two types of molecules. A higher antioxidant effect due to the presence of a galloyl group was expected because of the enhanced ability to release hydrogen atoms, which leads to an increased antioxidant effect and has already been shown in the literature [225, 273, 289]. However, in other studies, it had been shown, too, that a galloyl group can lead to a decreased antioxidant effect [225, 257, 258, 273]. Galloyl groups can lead to the formation of superoxide anions, which form stable radicals, which is why substances with a galloyl group often show a prooxidant effect [281]. Another possibility is steric hindrance due to the galloyl group, which would also result in a lower antioxidant effect. However, since the two oil methods differed mainly in the temperature used for the measurement, it also could be possible that substances with a galloyl group had a higher temperature stability and the effect in the P-DSC measurements was therefore higher. If a C7-OH group influences the result in the oil was not clear from this study, so further research was needed. However, it was already known from the literature that a C7-OH group also can positively affect antioxidant effect, so it was assumed that this effect also can be shown for rapeseed oil.

In both measurement methods, there was no negative influence of a **sugar residue** at position C6' of the dihydrochalcones, which is why one assumed that this observation also can be transferred to other subgroups. Naringin, which has two sugar residues at C7, showed no effect in the rancimat method. The reason could not be clarified in this study and requires further research. Maybe there is a negative effect of the sugar residue, but it is more likely that the molecule was sterically hindered, since the substance also showed a very little antioxidant effect in the P-DSC measurements. The presence of an additional **methoxy group** did not show an effect on the antioxidant activity neither in the rancimat method nor in the P-DSC measurements, which was not consistent with the literature [225, 273]. If a substance had a methoxy group instead of a hydroxyl group, a reduction in the antioxidant effect was determined in both measurement methods. Unlike the *in vitro* methods, there was no difference for the oil using substances with **hydroxycinnamic or hydroxybenzoic acids** groups. Therefore, the extension of the electron delocalization system did not seem to have any influence on the effect of the phenolic substances in the oil. According to the literature, hydroxycinnamic acids should have a higher antioxidant effect due to their additional conjugated double bond, as this biding increases the stability of the radical due to the extended electron delocalization [260, 269, 286, 287]. Furthermore, any influence of the **three-dimensional arrangement** on the antioxidant effect of the phenolic substances in the oil could be excluded, as this showed no effect in this study.

In summary, the results of the two oil measurement methods agreed quite close. In contrast to the *in vitro* assays, application-based methods did not require the use of model radicals. In the oil measurement methods, radicals are generated by the use of temperature, pressure, or oxygen, which then enter into a reaction with the antioxidants. Slight differences in the two methods can be attributed to the different temperatures and other measurement parameters, as these can influence the stability of antioxidants [291].

After performing the different *in vitro* antioxidant assays as well as the application-based oil measurement methods and investigating the structurally essential properties, which are important for a high antioxidant effect, all methods were compared. Thereby it should be possible to make a statement about the suitability of the *in vitro* assays for the prediction of the antioxidative effect of phenolic compounds in rapeseed oil.

5.6 Are in vitro assays suitable to predict the antioxidant activity of polyphenols in rapeseed oil?

Application-related measurement methods for determining the oxidation stability of lipophilic samples are often time-consuming and complex and are therefore associated with increased cost. For this reason, there is a need for cheap, simple and rapid methods, which allow a first statement about the antioxidant behavior in oil [43]. One knew already from the literature that the ORAC assay is suitable for measuring lipophilic samples and should correlate with results in oil [206, 252]. In addition, another study shows a correlation between the oxidative stability of nut oil for the DPPH assay with oil stability tests. However, no antioxidants are used here [292]. Since there are only a few literature studies in this area, continued research was needed to fill these gaps.

This thesis initially revealed that both the *in vitro*, and the oil measurement methods are suitable for determining the antioxidant activity of phenolic compounds. All methods were influenced by structural properties, which are responsible for high antioxidant activity. However, the analyses showed, that the methods differ among themselves, which indicated that they were influenced differently by structural properties, as described and discussed above in detail. The results of the PC analyses suggested that the ORAC, ABTS, and FC assay may react according to the same or similar reaction mechanism, and therefore these assays correlate with each other. However, it should be noted that especially the ORAC and ABTS assay were similar. Since these two assays are classified in the literature as belonging to both the SET- and HAT-based assays, they may underlie the same reaction mechanism [251–256]. Furthermore, the PC analysis showed a correlation of the DPPH assay with the two oil measurement methods, thus indicating the suitability of this assay for the prediction of antioxidant activity in oil. The literature already showed a correlation of results in the DPPH assay with the oxidative stability of nut oil. However, nut oil was tested without the addition of antioxidants here [292].

Further PC analyses found that for the flavonoids, in addition to the DPPH assay, the FC assay was suitable for predicting antioxidant activity in the oil, but neither the ABTS nor the ORAC should be used. The phenolic acids showed a different result. Here, the two oil measurement methods showed no correlation. Again, a possible reason for this could be the different parameters during the measurement. The phenolic acids may be specifically sensitive to temperature, which led to different results. Maybe, the results of the rancimat method, in which the gentler parameters were chosen compared to the P-DSC, should be more emphasized. Since the PC analysis showed the strongest correlation between the rancimat method and the DPPH assay, this method should also be used preferentially for phenolic acids to predict the antioxidant activity in the oil.

In summary, the PC analyses confirmed that the results of the measurement methods partly depend on different structural properties and thus may be subject to variable reaction mechanisms. Despite these findings, a correlation between the oil measurement methods and the DPPH assay was found. For flavonoids, phenolic acids, and a mixture of flavonoids, and phenolic acids. In addition, there was a correlation, even though lower, between the FC assay and the oil measurement methods.

Thus, this study demonstrated that two of the *in vitro* assays are potentially helpful for predicting the antioxidant activity of phenolic components in oil. Both the benefit from these findings and what further research is still needed will be discussed in the following. Furthermore, it will be discussed whether the use of phenolic compounds as antioxidant additives is suitable and what advantages and disadvantages they entail.

5.7 Impact on the lubricant industry and need for further research

The need for totally bio-based lubricants has increased significantly in recent years [17–22]. Since naturally occurring phenolic compounds are known for their high antioxidant activity, they are potentially suitable as alternatives [23–29]. In addition, they are increasingly found in residual materials from the agricultural and food industries and thus can be acquired inexpensively and obtained by solvent-based extraction [22–29, 38]. Accordingly, further research was needed to determine whether the phenolic components contained are potentially suitable to replace conventional antioxidant additives.

This thesis showed that phenolic components from the subclasses of phenolic acids, flavonols, flavanones, dihydrochalcones, and flavanols could increase the oxidative stability of rapeseed oil up to 230 % and are therefore potentially suitable for a protection against oxidation. However, further research is required before a statement can be made about their suitability as additives for lubricants. For example, the compatibility of the phenolic compounds to other lubricant additives should be tested to ensure that they are not adversely affected or precipitate.

Since most lubricants are not based on vegetable oils, compatibility with other base fluids should also be tested. Furthermore, it should be investigated whether the phenolic compounds have a negative influence on other typical lubricant properties, such as the influence on wear and friction as well as corrosion. This investigation requires tribological and rheological measurements and an analysis of corrosion resistance.

Since phenolic standard references are very expensive, residual materials from the agricultural and food industries, the latter should be used to obtain the phenolic components in order to save costs. In this case, tests must necessarily prove whether the extracts in their original form are suitable for use in lubricants or whether they have to be purified beforehand. Ingredients such as sugars, carbohydrates, and acids could negatively influence the stability and the properties of the lubricants.

As shown in this thesis, it is possible to predict the effect of phenolic substances in rapeseed oil by using *in vitro* assays. Thus, suitable substances could be pre-selected before carrying out cost-intensive application tests. However, before the *in vitro* methods can be applied in the lubricant field, further research is needed, for example, to verify whether the results of the methods correlate only with the use of rapeseed oil or also other oils. For this purpose, it is necessary to incorporate the phenolic substances used into other desired base media and repeat the experiments. In measure the results of phenolic-rich extracts, it is also necessary to check the influence of the sample matrix on the results of the *in vitro* assays as well as the oil measurement methods.

6 Summary

According to the current state of the art, most lubricants and additives are based on mineral oil and are classified as hazardous to health and environment. Vegetable oils represent a possible alternative, but their use is currently not possible due to their low oxidation stability. A bio-based alternative to increase the oxidation stability is natural antioxidants, which are partly contained in large quantities in waste materials and residues from the agriculture and food industry. Most of these are phenolic substances, ranging from the low-molecular-weight phenolic acids to the more complex flavonoids. Usually, the phenolic substances can be obtained by a simple solvent-based extraction.

To determine the antioxidant activity and polyphenolic content of phenol-rich extracts, a range of *in vitro* assays have been developed. Among the most commonly used are the ABTS, DPPH, FC, and ORAC assay. Although there are already many studies on these assays and their use to determine the antioxidant activity, these do not indicate the type of phenolic substances and thus the sort of extracts for which these assays are suitable. In addition, there are different measurement and evaluation methods, which makes it difficult or even impossible to compare studies from the literature with statements about the structure-activity relationships between the phenolic substances and the model radicals used in the assays. Also, comparing between the results of the *in vitro* assays with application-related test methods was not sufficiently described before. Thus, there is no statement if *in vitro* assays can be used to predict antioxidant activity in oils. However, this would be a valuable information, especially for the technical fields, where expensive and complex oil measurement methods are performed. Therefore, this work aimed to fill the mentioned gaps in the literature regarding *in vitro* antioxidant assays and to enable the use of natural phenolic substances as alternative antioxidant additives in lubricants.

First, various *in vitro* antioxidant assays were investigated as to their structure-activity relationships and the structural properties responsible for high antioxidant activity. Both HAT- (ORAC) and SET-based assays (ABTS, DPPH, FC) were applied, and, for the comparison, the same concentrations of phenolic substances and, as far as possible, the same measurement and evaluation methods were used.

By adapting the measurement methods, it was possible to determine the antioxidant effect of the phenolic substances as kinetics over time. Due to different reaction rates, the substances needed different amounts of time to reach the end point of the reaction. By adopting second-order reaction kinetics and extrapolating the measurement curves, it was possible to calculate the final values and shorten the experimental measurement duration from several hours to 30 min without underestimating the antioxidant effect of the substances.

Despite the adapted methods and the use of the same concentrations of phenolic substances, the assays showed different trends in direct comparison. The results depended, to varying degrees, on structural properties such as the Bors criteria and the number and positions of the hydroxyl groups and other substituents. Yet overall, compounds fulfilling all three Bors criteria achieved the highest antioxidant activity in the ABTS, DPPH, and FC assay. In the ORAC assay, on the other hand, the number of hydroxyl groups was critical for a high effect.

In addition, it was possible to identify commonalities among the four assays by statistical evaluation of the results using PC analysis. Each assay correlated with antioxidant activity and is therefore suitable for its measurement, but that they base upon on different reaction mechanisms. Based on their correlations, both the ORAC and the ABTS, as well as the FC and the DPPH assay, follow similar reaction mechanisms, respectively.

Furthermore, it was shown that the antioxidant effect depends on the Bors criteria and the number of hydroxyl groups. The highest correlation was showed for the FC assay. The highest correlation of these properties showed for the FC assay being the only assay that bases upon a reduction reaction of the FC reagent but not the use of a model radical. Therefore, the steric hindrance of the model radicals, which can distort the result, did not play a role.

Studying the antioxidant effect in oil, phenolic substances of the same subgroups were incorporated into rapeseed oil, determining their impact by the rancimat method and P-DSC. Thus, it was possible to increase the oxidative stability of rapeseed oil up to approx. 230 %, showing that polyphenols are potentially suitable for use in lubricants. However, further research is needed before they can actually be used, as, for example, compatibility with other additives must be tested. In addition, PC analyses were performed to identify possible correlations with the *in vitro* assays. These revealed a high correlation of the DPPH assay with the two oil measurement methods. The results of these three methods were again affected by the Bors criteria and the number of hydroxyl groups. Advantages of the DPPH assay, besides the small sample size, are the ease of performance and the fact that the assay can be performed with simple laboratory equipment and without extensive training. For the flavonoid group, the oil measurement methods also showed correlations with the FC assay. Thus, depending on the composition of the sample to be measured, both the FC and the DPPH assay are possible alternatives to replace the time-consuming and complex oil measurement methods. However, it needs more research before the *in vitro* methods can be established in the lubricant field. For example, it should be clarified if a correlation can also be shown for other vegetable oils. It is also important to note that the methods have only been tested for using phenolic standard references and their suitability for measuring extracts and the correlation of these results to oil measurement methods first needs to be verified. To transfer the methods to phenolic extract mixtures, it is necessary, in addition, to investigate the influence of the sample matrix (sugar, protein, acid content) on the measurement results.

7 Zusammenfassung

Nach Stand der Technik sind die meisten Schmierstoffbasismedien und Additive mineralölbasiert und werden als gesundheitsgefährdend und krebserregend eingestuft. Eine mögliche Alternative stellen pflanzliche Öle dar, deren Einsatz auf Grund der geringen Oxidationsstabilität derzeit allerdings noch nicht möglich ist. Eine biobasierte Möglichkeit die Oxidationsstabilität zu erhöhen, stellen natürlichen Antioxidantien dar, welche teilweise in großen Mengen in Nebenströmen der Agrar- und Lebensmittelindustrie enthalten sind. Meist handelt es sich hierbei um phenolischen Substanzen, welche von den niedermolekularen Phenolsäuren bis hin zu den komplexeren Flavonoiden reichen. In der Regel können die phenolischen Substanzen durch eine einfache lösungsmittelbasierte Extraktion gewonnen werden.

Um die antioxidative Wirkung und den Polyphenolgehalt von phenolreichen Extrakten zu bestimmen, wurde eine Vielzahl von *in vitro* Assays entwickelt. Zu den am häufigsten verwendeten zählen der ABTS, DPPH, FC und ORAC Assay. Obwohl es bereits viele Studien zu diesen Assays und ihrer Verwendung zur Bestimmung der antioxidativen Wirkung gibt, geht aus ihnen nicht hervor, für welche Art von phenolischen Substanzen und somit auch für welche Art von Extrakten sich welcher der Assays am besten eignet. Außerdem gibt es unterschiedliche Mess- und Auswertemethoden, was einen Vergleich zu Studien aus der Literatur und Aussagen über die Struktur-Aktivitäts Beziehungen zwischen den phenolischen Substanzen und den verwendeten Modellradikalen der Assays, schwierig oder sogar unmöglich macht. Auch ein Vergleich zwischen den Ergebnissen der *in vitro* Assays mit anwendungsbezogenen Testmethoden wurde in der Literatur nicht ausreichend beschrieben. So wurde bisher keine Aussage darüber getroffen, ob sich die *in vitro* Assays zur Vorhersage der antioxidativen Wirkung in Ölen verwenden lassen. Dies wäre jedoch besonders für die technischen Bereiche eine wertvolle Information, da hier teure und aufwendige Ölmessmethoden durchgeführt werden. Ziel dieser Arbeit war es daher, die genannten Wissenslücken im Bezug auf die *in vitro* antioxidativen Assays zu schließen und den Einsatz von natürlichen, phenolischen Substanzen als alternative Oxidationsschutz-Additive im Schmierstoffbereich zu ermöglichen.

Zunächst wurden verschiedene *in vitro* Assays hinsichtlich ihrer Struktur-Aktivitäts-Beziehungen untersucht sowie die strukturellen Eigenschaften identifiziert, welche für eine hohe antioxidative Wirkung im jeweiligen Assay verantwortlich sind. Es wurden sowohl HAT (ORAC) als auch SET basierte Assays (ABTS, DPPH, FC) verwendet und für den Vergleich die selben Konzentrationen an phenolischen Substanzen und, soweit möglich, gleiche Mess- und Auswertemethoden verwendet.

Durch die Anpassung der Messmethoden war es möglich, die antioxidative Wirkung der phenolischen Substanzen als Kinetik über die Zeit zu bestimmen. Aufgrund unterschiedlicher Reaktionsgeschwindigkeiten benötigten die Substanzen unterschiedliche Zeiten bis sie den Endpunkt der Reaktion erreichten. Durch die Adaption der Reaktionskinetik zweiter Ordnung und Extrapolation der Messkurven, war es möglich, die Endwerte zu berechnen und die experimentelle Messdauer von mehreren Stunden auf 30 min zu verkürzen, ohne die antioxidative Wirkung der Substanzen falsch zu interpretieren.

Trotz der angepassten Methoden und der Verwendung der gleichen Konzentration an phenolischen Substanzen, zeigten die Assays im direkten Vergleich unterschiedliche Trends. Dabei hingen die Ergebnisse unterschiedlich stark von strukturellen Eigenschaften wie den Bors Kriterien und der Anzahl und Positionen der Hydroxylgruppen und anderer Substituenten ab. Insgesamt erzielten aber Substanzen, welche alle drei Bors Kriterien erfüllen, sowohl im ABTS als auch im DPPH und FC Assay die höchste antioxidative Wirkung. Im ORAC Assay hingegen, war die Anzahl der Hydroxylgruppen entscheidend für eine hohe Wirkung. Zusätzlich war es durch statistische Auswertung der Ergebnisse in Form einer PC Analyse möglich Gemeinsamkeiten der vier Assays zu identifizieren. Es konnte gezeigt werden, dass jeder der Assays mit der antioxidativen Wirkung korreliert und daher für deren Messung geeignet ist, die Assays aber auf unterschiedlichen Mechanismen beruhen. Aufgrund der Korrelationen zwischen dem ORAC mit dem ABTS sowie dem FC mit dem DPPH Assay wird vermutet, dass diesen jeweils ähnliche Reaktionsmechanismen zugrunde liegen, die sich von den beiden Anderen unterscheiden.

Desweiteren konnte eine Abhängigkeit der antioxidativen Wirkung von den Bors Kriterien und der Anzahl der Hydroxylgruppen gezeigt werden. Die höchste Korrelation dieser Eigenschaften zeigte sich für den FC Assay. Hierbei handelt es sich um den einzigen Assay, der nicht auf der Verwendung eines Modelradikals, sondern auf einer Reduktionsreaktion des FC Reagenz basiert. Daher spielten in diesem Assay sterische Hinderungen der Modellradikale, welche das Ergebnis verfälschen können, keine Rolle.

Zur Untersuchung der antioxidativen Wirkung im Öl wurden phenolische Substanzen der gleichen Untergruppen in Rapsöl eingearbeitet und deren Wirkung mittels Rancimat Methode und P-DSC bestimmt. So war es möglich, die Oxidationsstabilität des Rapsöls um bis zu ~230 % zu erhöhen, was die Substanzen potentiell für den Einsatz in Schmierstoffen geeignet macht. Bevor diese jedoch tatsächlich eingesetzt werden können, bedarf es weiterer Forschung, da beispielsweise die Verträglichkeit mit anderen Additiven getestet werden muss. Zusätzlich wurden PC Analysen durchgeführt um mögliche Korrelationen mit den *in vitro* Assays zu identifizieren. Diese Analysen ergaben eine gute Korrelation des DPPH Assays mit den beiden Ölmessmethoden. Auch die Ergebnisse dieser drei Methoden wurden von den Bors Kriterien sowie von der Anzahl der Hydroxylgruppen bestimmt.

Vorteile des DPPH Assays sind neben der kleinen Probenmenge, die einfache Durchführung, sowie die Tatsache, dass der Assay mit simplen Laborgeräten und ohne aufwendige Schulungen durchgeführt werden kann. Für die Gruppe der Flavonoide zeigte sich zudem eine Korrelation der Ölmessmethoden mit dem FC Assay. Je nach Zusammensetzung der zu messenden Probe, bieten sich also sowohl der FC als auch der DPPH Assay als mögliche Alternative an, um die zeitaufwendigen und komplexen Ölmessmethoden zu ersetzen.

Bevor die *in vitro* Methoden jedoch im Schmierstoffbereich etabliert werden können, bedarf es noch weiterer Forschung. Es sollte beispielsweise geklärt werden, ob eine Korrelation auch für andere pflanzliche Öle gezeigt werden kann. Es gilt außerdem zu beachten, dass die Methoden nur für die Verwendung von phenolischen Reinsubstanzen getestet wurden und ihre Eignung zur Messung von Extrakten und die Korrelation dieser Ergebnisse zu den Ölmessmethoden erst überprüft werden müsste. Um die Methode auf phenolische Extraktmischungen zu übertragen, ist es zusätzlich nötig den Einfluss der Probenmatrix (Zucker-, Protein, Säuregehalt) auf das Messergebnis zu untersuchen.

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