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Genotypic and Climatic Influence on the Antioxidant Activity of Flavonoids in Kale (*Brassica oleracea* var. *sabellica*)

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The influence of genotype and climatic factors, e.g. mean temperature and mean global radiation level, on the antioxidant activity of kale was investigated. Therefore, eight kale cultivars, hybrid and traditional, old cultivars, were grown in a field experiment and harvested at four different times. In addition to the investigation of the total phenolic content, the overall antioxidant activity was determined by TEAC assay and electron spin resonance spectrometry. A special aim was to characterize the contribution of single flavonoids to the overall antioxidant activity using an HPLC−online TEAC approach. The antioxidant activity and the total phenolic content were influenced by the genotype and the eco-physiological factors. The HPLC−online TEAC results showed that not all flavonol glycosides contribute to the overall antioxidant activity in the same manner. Taking the results of the structural analysis obtained by HPLC−ESI−MS^n into account, distinct structure−antioxidant relationships have been observed.

KEYWORDS: Curly kale; temperature; global radiation; total phenolic content; HPLC−online TEAC; structure−activity relationship; flavonoid glycosides

INTRODUCTION

Brassica plants, such as *Brassica oleraceae* sub species (e.g., cauliflower, cabbage, Brussels sprout, broccoli) and *Brassica rapa* subspecies (e.g., turnip, pak choi) are major vegetable crops grown throughout the world. *Brassica oleracea* var. *sabellica* (kale) for instance, is a leafy cabbage commonly cultivated in Germany and other places in Central and Northern Europe during winter but also in North Africa, the USA, and parts of South America. With regard to the human diet, kale is an important source of high amounts of vitamins and natural antioxidants (*1*), including ascorbic acid (92.6−186 mg 100 g^{-1} edible portion) as well as β-carotene (2.84−9.23 mg 100 g^{-1} edible portion) (*2*). As a typical representative of the *Brassica* family, kale is also rich in secondary plant metabolites, such as flavonoids. Kale's flavonoids are comprised mainly of flavonol derivatives, which are reported to consist of various glycosides of isorhamnetin, quercetin, or kaempferol that are often acylated with hydroxycinnamic acids (*3*−*5*). The concentration as well as the composition of flavonoids varies due to cultivar, maturity at harvest, soil and water state, climate (e.g., temperature and radiation level), and postharvest treatments (*2*,*4*,*6*−*8*), all aspects that influence the biosynthesis of flavonoids.

During the last 20 years, the flavonoids have gained much attention due to their beneficial health potential, which is thought to be related to the antioxidant activity of the flavonoids. Because of their antioxidant character, resulting from different conjugations and varying numbers of hydroxyl groups, flavonoids and phenolic acids are able to act as reducing agents, as hydrogen- or electron-donating species, and as reactive oxygen species (ROS) scavengers (*9*). A large number of studies investigating the overall antioxidant activities among different *Brassicaceae*, including mostly Brussels sprouts, cauliflower, cabbage, and broccoli, have been published during the last few years (*2*). A few of these also include kale as investigated *Brassica* species. Out of the enormous number of articles, only a few have investigated the influence of genotype and/or climate on the antioxidant activity of a single *Brassica* vegetable. To our knowledge only two of these studies centered on kale. Hagen et al. reported a 25−30% decrease of the overall antioxidant activity of kale cultivated at low temperatures (*8*). Martuza et al. reported a higher total phenolic content for wild kale genotypes compared to cultivated genotypes (*10*).

Due to the large number of antioxidant components in plants, many screening methods for the determination of the total antioxidant capacity of polyphenols, all based on their radical scavenging activities, have been reported and reviewed (*11*, *12*). These methods include the trolox equivalent antioxidant capacity assay (TEAC assay), the oxygen radical absorbance capacity assay (ORAC assay), the 1,1-diphenyl-2-picrylhydrazyl assay (DPPH radical assay), and electron spin resonance spectrometry (ESR). All of these methods detect the overall antioxidant activity; however, they are not useful for estimating the contribution of single components. A general practice to assess the antioxidant capacity of single components is separating and purifying them, which is time-consuming and produces a lot of waste. Therefore,

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more elaborate techniques are required. Recently, a simultaneous determination of structure and antioxidant behavior using HPLC coupled directly (online) to the TEAC assay was reported for tea (*13*−*15*).

The purpose of the present study was to assess the overall antioxidant activity and total phenolic content of eight kale cultivars, including red and green kale as well as old, traditional, and hybrid cultivars. Furthermore, the influence of eco-physiological factors during cultivation such as mean temperature and mean global radiation was investigated. Therefore, the TEAC assay as a well-established, easy to use method comparable to other researchers' results was used. For the reason of comparison and assurance of the TEAC results, ESR spectrometry was used as a reference method. An important advantage of this method is the matrix-independent measurement of the reaction between antioxidants and radicals in an electromagnetic field instead of a photometric absorbance. A special aim of this study was to characterize the contribution of single flavonoids to the overall antioxidant activity using an HPLC−online TEAC approach. This is the first time this method has been applied to a *Brassica* vegetable.

MATERIALS AND METHODS

Chemicals. ABTS (2,2′-Azinobis(ethylbenzothiazoline-6-sulfonic acid); ∼ 98%), potassium persulfate (≥99%), trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; 97%), Folin−Ciocalteu's phenol reagent, and Fremy's salt were purchased from Sigma-Aldrich (Steinheim, Germany); sodium chloride p.a., sodium carbonate p.a., dipotassium hydrogen phosphate p.a., potassium dihydrogen phosphate p.a., and methanol were purchased from Merck (Darmstadt, Germany); gallic acid monohydrate (99.5%) was purchased from Serva (Heidelberg, Germany); acetonitrile and acetic acid p.a. (100%) were purchased from Roth (Karlsruhe, Germany). All solvents were of HPLC grade, and water was of Milli-Q quality.

Plant Material. To analyze the genotypic influence, eight kale cultivars were set in a randomized block design with three replicates at the experimental fields of the Leibniz Institute of Vegetable and Ornamental Crops Grossbeeren/Erfurt e.V. (Grossbeeren, Germany): F1 hybrids 'Winterbor' (Bruno Nebelung, Norken, Germany), 'Redbor' (a red kale cultivar) (Chrestensen, Erfurt, Germany), 'Winnetou' (Bruno Nebelung, Norken, Germany), and 'Arsis' (Gärtner Pötschke, Kaarst, Germany); traditional, old cultivars 'Altmärker Braunkohl' (with red leaves) (Dulcamara-Samen, Carmzow-Wallmow, Germany), 'Halbhoher grüner Krauser' (Chrestensen, Erfurt, Germany), and 'Lerchenzunge' (Gärtner Pötschke, Kaarst, Germany); 'Frostara' (Bruno Nebelung, Norken, Germany). The soil was loamy sand with 810 mg g^{-1} sand, 140 mg g^{-1} silt, and 50 mg g^{-1} clay. In the plow layer, the soil pH was 6.5, and the soil bulk density was 1.6 g cm^{-3}. The sowing date for all kale plants in the year 2007 was June 18th, and plants in the four-leaf stage were transplanted into the field on July 17th. Before planting, soil samples in the 0−30 cm soil profile were taken and analyzed for mineralized nitrogen. Nitrogen fertilizer was added in several applications (one basic dressing and five top dressings) to meet the N requirement of 200 kg ha^{-1} of mineralized nitrogen. Irrigation was controlled with the help of the "BEREST" program (*16*). Kale plants were also covered by protection nets against pests until the middle of September. Thus, the influence of cultivation was standardized, enabling the determination of genotypic and climatic effects on the concentration of phenolic compounds as well as on the antioxidant activity. Per replication, six fully developed plants of each cultivar were harvested four times in 4 week intervals (October 2007, November 2007, December 2007, and January 2008). The daily mean temperature and the daily mean global radiation were calculated from measurements taken every 2 and 5 min, respectively, during the 4 weeks before each harvest (October 2007, 9.7 °C, 713 μmol m^{-2} s^{-1}; November 2007, 6.0 °C, 285 μmol m^{-2} s^{-1}; December 2007, 3.8 °C, 179 μmol m^{-2} s^{-1}; January 2009, 0.3 °C, 172 μmol m^{-2} s^{-1}).

Extraction of the Phenolic Compounds. Each cultivar replication was investigated in duplicate. Therefore, at each harvest time, a mixture of leaves (without the middle rib) taken from six plants was frozen (−40 °C), lyophilized, and ground. For extraction, 2 g of ground sample was dissolved in 25 mL of 62.5% aqueous methanol and stirred at 500 rpm for 1 h. The mixture was then filtered, and aliquots were used for further analysis.

Total Phenolic Content. The total phenolic content of kale extracts was determined using the Folin−Ciocalteu colorimetric method (*17*), with little modifications. In brief, 400 μL of a 20-fold dilution of each extract was mixed with 2.5 mL of distilled water, 1 mL of Na_2CO_3 (7.5% w/v), and 100 μL of Folin−Ciocalteu reagent. The thoroughly mixed solution was incubated at 35 °C for 15 min. The absorbance was measured at 736 nm (SPECORD 40, Analytik Jena AG, Jena, Germany) after the solution had cooled to room temperature. The results were expressed as millimoles of gallic acid equivalents per gram of dry matter (mmol GAE g^{-1} dm). All extracts were analyzed in duplicate.

Trolox Equivalent Antioxidant Capacity Assay. The ability to scavenge the $ABTS^{\bullet+}$ radical was determined using the TEAC assay modified by Rohn et al. (*18*). Shortly thereafter, 100 μL of a 25-fold dilution of the methanol extract was mixed with 500 μL of an ABTS working solution (500 μM). Exactly 6 min after adding 200 μL of $K_2S_2O_8$ (10 mM) to the mixture, the absorbance was measured at 734 nm (SPECORD 40, Analytik Jena AG, Jena, Germany). Results were expressed as millimoles of trolox equivalents per gram of dry matter (mmol trolox g^{-1} dm). Each extract was investigated in duplicate.

Electron Spin Resonance Spectrometry. The antioxidant activity of the kale cultivars against Fremy's salt was measured by ESR spectrometry. Therefore, 100 μL of a 50-fold dilution of the extracts was allowed to react with 100 μL of a 1 mM Fremy's salt solution in phosphate buffer. The ESR spectrum of Fremy's radical was recorded after 15 min. The intensity was obtained by integration of the signal. The antioxidant activity, expressed as millimoles of Fremy's salt per gram dry matter (mmol Fremy's salt g^{-1} dm), was calculated in comparison to a control reaction with distilled water. Spectra were recorded at 21 °C on a Miniscope MS 100 spectrometer (Magnettech GmbH, Berlin, Germany). The microwave power and modulation amplitude were set at 10 dB and 1500 mG, respectively.

Antioxidant Capacity by HPLC−UV/vis−Online TEAC. For chromatographic separation, 4 mL of methanolic kale extract was dried under nitrogen and redissolved in 900 μL of 0.5% acetic acid in water, with 100 μL of a 10 mM trolox solution added as internal standard (final concentration 1 mM trolox). Prior to injection the sample was filtered through a 0.45 μm Nylon syringe filter. The HPLC system consisted of a quaternary HPLC pump (Shimadzu LC-10ATvp), a degasser (Shimadzu DGU-20A), a gradient mixer (Shimadzu FCV-10ALvp), a UV/vis detector (Shimadzu SPD-10Avp) set at 280 and 370 nm, an autosampler (Shimadzu SIL-10AF), a column oven (Shimadzu CTO-10ASvp) set at 30 °C, and a second pump (Shimadzu LC-10ATvp) pumping the $ABTS^{\bullet+}$ working solution (all devices Shimadzu Europa GmbH, Duisburg, Germany). According to Schmidt et al. (*5*) the separation was carried out on a Prodigy column (150 × 3.0 mm, ODS 3, 5 μm, 100 Å; Phenomenex Ltd., Aschaffenburg, Germany) with a security guard C18 (4 × 3.0 mm) using a gradient of (A) 0.5% acetic acid in water and (B) 100% acetonitrile. The following gradient was used: 5−7% B (0−12 min), 7−9% B (12−25 min), 9−12% B (25−45 min), 12−15% B (45−100 min), 15% B isocratic (100−150 min), 15−28% B (150−180 min), 28−50% B (180−220 min), 50% B (220−230 min), 50−5% B (230−240 min), 5% B (240−245 min). The flow rate was set to 0.4 mL min^{-1} (*5*).

The antioxidant activity of the kale extracts was determined using the HPLC−DAD−online TEAC assay described by Stewart et al. and Koleva et al. (*13*, *14*). A 2 mM $ABTS^{\bullet+}$ stock solution containing 3.5 mmol of potassium persulfate was incubated overnight at room temperature in the dark to allow for stabilization of the radical. The $ABTS^{\bullet+}$ working solution was prepared by diluting the stock 10-fold in phosphate buffer.

Aliquots of the methanolic kale extracts were injected, and separation was carried out as described above. The eluate from the HPLC−UV detector was mixed with the $ABTS^{\bullet+}$ working solution using a T-valve and a reaction capillary (325 × 0.7 mm). The $ABTS^{\bullet+}$ working solution was pumped into the T-valve using the second pump with a flow set to 0.3 mL min^{-1}. Compounds of the HPLC eluate that were capable of scavenging the $ABTS^{\bullet+}$ led to a decolorization of the colored $ABTS^{\bullet+}$ working solution, which resulted in a negative peak. The negative peaks are related to the positive ones in the normal HPLC chromatogram. The decolorization of $ABTS^{\bullet+}$ was measured with a second UV/vis detector (Shimadzu

SPD-10Avp) at 600 and 414 nm. The data were analyzed using LC Lab Solution 10 Software (Shimadzu Europa GmbH, Duisburg, Germany). The antioxidant activity of single compounds was calculated as trolox equivalents (TE) related to the area under the curve at 414 nm produced by a 1 mM trolox solution as internal standard. The antioxidant capacity as well as the antioxidant potential of the single compounds were referenced to the ratio of the area at 414 and 280 nm, respectively.

Structural Investigation and Characterization of the Phenolic Compounds by HPLC−DAD−ESI-MS^n. To determine the flavonoid glycosides, an HPLC Series 1100 instrument from Agilent (Waldbronn, Germany) consisting of degasser, binary pump, auto sampler, thermostat, and photodiode array detector was used. Except for the instrumentation, the chromatographic conditions were the same as those described above. The extracts were separated on a Phenomenex Prodigy (125 × 3.0 mm, ODS 3, 5 μm, 100 Å) column with a security guard C18 (4 × 3.0 mm) at a temperature of 30 °C using the water/acetonitrile gradient described previously (*5*). The flavonoid glycosides were identified as deprotonated molecular ions and characteristic mass fragment ions by HPLC−DAD−ESI-MS^n using an Agilent Series 1100 ion trap in negative ionization mode. Nitrogen was used as dry gas (12 L min^{-1}, 350 °C) and nebulizer gas (40 psi) at a capillary voltage of ~3500 V. Helium was used as collision gas in the ion trap. The mass optimization was performed for quercetin at m/z 301 and arbitrary for m/z 1000. The MS^n experiments were performed in auto or manual mode until MS^4 in a scan from m/z 200 to 2000.

Statistical Analysis. Data in figures were reported as mean ± SD for triplicate analyses, while all other data were reported just as the mean. For the analysis of variance (two-way ANOVA), Tukey's Honest Significant Difference (HSD) test was used to calculate significant differences at a significance level of $p \leq 0.05$. Analysis of variance was performed to test for significance of cultivar, harvest time, and cultivar × harvest time by using Statistica (version 9.0, Statsoft Inc., Tulsa, OK). A Pearson Correlation test was conducted to determine the correlations among means ($p \leq 0.05$).

RESULTS AND DISCUSSION

Influence of Genotype on the Total Phenolic Content. Eight kale cultivars harvested at four times in 4 week intervals between October 2007 and January 2008 were analyzed using the Folin−Ciocalteu assay to determine the genotypic influence on the total phenolic content (TPC) in relation to all harvest dates. The gallic acid equivalent concentration (GAE) ranged between 0.18 and 0.31 mmol g^{-1} dry matter (dm), which is related to 3.15−5.86 mmol 100 g^{-1} fresh weight (fw) (**Figure 1A**). As seen in **Figure 1A** the genotypic variation shows that especially the traditional, old cultivars 'Altmärker Braunkohl', 'Halbhoher grüner Krauser', and 'Lerchenzunge' as well as the red hybrid 'Redbor' are characterized by relatively high total phenolic content, while the cultivars 'Winterbor', 'Frostara', 'Winnetou', and 'Arsis' have lower values. Together with 'Altmärker Braunkohl' 'Redbor' has the highest TPC of all investigated kale cultivars: 0.29 and 0.31 mmol GAE g^{-1} dm, respectively.

The investigated genotypes of the present study showed higher TPC values compared to those found by other researchers, who obtained values ranging from 0.08 to 0.11 mmol GAE g^{-1} dm (*1*, *3*, *8*, *19*). Also comparable to the actual results, Sikora et al. determined 2.18 mmol chlorogenic acid equivalents per 100 g fw for the cultivar 'Winterbor' (*20*), whereas Ayaz et al. determined just 0.80 μmol GAE 100 g^{-1} fw (*21*).

In a study from 2005, Borge et al. determined a higher TPC for 'Redbor' than for the green kale 'Ekstra Moskruset' (*22*). Moreover, recent results of Schmidt et al. showed that although 'Redbor' contains a similar total aglycone concentration in comparison to the other cultivated hybrid genotypes, its quercetin concentration is as high as in the old, traditional genotypes 'Altmärker Braunkohl', 'Halbhoher grüner Krauser', and 'Lerchenzunge' (*4*), assuming that either further phenolic compounds or quercetin itself contributes dominantly to the high TPC in 'Redbor'.

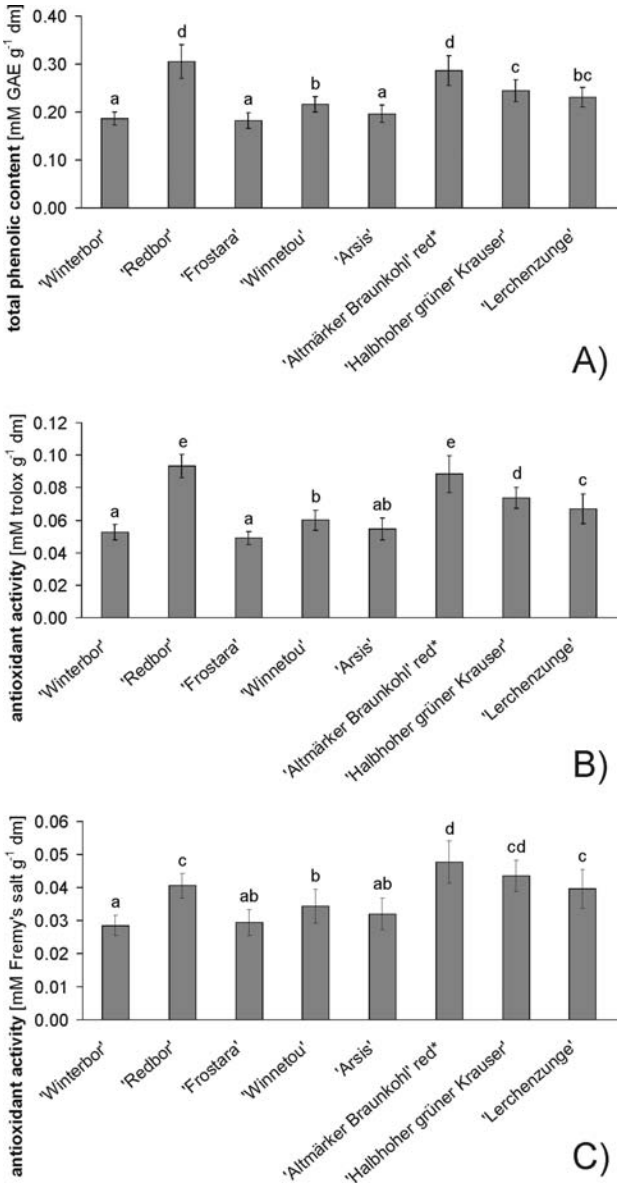

Figure 1. Genotypic effect on the total phenolic content (**A**), and the antioxidant activity (measured with TEAC (**B**) and ESR (**C**)) of eight kale cultivars in relation to four harvest times in a 4 week interval between October 2007 and January 2008. Results were expressed as millimoles of gallic acid equivalents per gram of dry matter (**A**), as millimoles of trolox equivalents per gram of dry matter (**B**), and as millimoles of Fremy's salt per gram of dry matter (**C**). Different letters in each chart indicate significant differences between the cultivars ($p \leq 0.05$ by Tukey's HSD test). Asterisks denote varieties that have a red leaf color ($n = 3$).

Influence of Genotype on Trolox Equivalent Antioxidant Capacity Assay. The eight kale cultivars harvested between October 2007 and January 2008 were analyzed using the trolox equivalent antioxidant capacity assay to determine the genotypic influence on antioxidant activity. In comparison to the results of the Folin−Ciocalteu assay, the results of the TEAC assay (**Figure 1B**) show a similar influence of the genotype on antioxidant activity. As before, the cultivars 'Redbor', 'Altmärker Braunkohl', 'Halbhoher grüner Krause', and 'Lerchenzunge' contain in descending order the highest antioxidant activity, whereas the other three hybrid cultivars and 'Frostara' showed lower values. It is obvious that the cultivars which have the highest TPC also have the highest antioxidant activity. This leads to the conclusion that the

TPC has a strongly positive correlation with the antioxidant activity, proven by a correlation factor of $p = 0.903$.

In summary, the antioxidant activity of the eight cultivars expressed in mmol trolox equivalents g^{-1} dm ranged between 0.049 and 0.093 mmol TE g^{-1} dm or 0.879−1.784 mmol TE 100 g^{-1} fw (**Figure 1B**).

The results of the red and green kale cultivars investigated by Borge et al. revealed a higher antioxidant activity (measured with the DPPH, FRAP, and ORAC assay) of 'Redbor' compared to the green kale 'Ekstra Moskruset' (*22*). In comparison to the results shown here, 'Redbor' has a 1.1−1.9 fold higher antioxidant activity related to the mainly green old, traditional and hybrid cultivars used in the present study.

Furthermore, Zhou et al. detected 0.059 mmol TE g^{-1} dm for the $ABTS^{\bullet+}$ scavenging activity of kale (*19*), which is comparable to the results shown in **Figure 1B**. However, Zhou et al. determined a lower TPC compared to the present study but also showed a direct correlation between the TPC and the $ABTS^{\bullet+}$ scavenging activity (*19*). Reporting a total phenolic content of 2.18 mmol of chlorogenic acid per gram fw, Sikora et al. detected 3.62 mmol TE 100 g^{-1} fw, an antioxidant activity more than 2 times higher than that found in the present study (*20*). The different values may arise from various extraction methods of the sample preparation, varying extractants, unequal points of measurement, and the numerous modifications of the TEAC and Folin−Ciocalteu assay among the researchers.

Influence of Genotype on the Antioxidant Activity Measured by Electron Spin Resonance. To compare the results from the TEAC and Folin−Ciocalteu assay, the eight different kale cultivars harvested from October 2007 to January 2008 were additionally analyzed using ESR with Fremy's salt as the radical. The influence of the genotype on the antioxidant activity was analyzed. The metabolized amount of Fremy's salt after 15 min of reaction time ranged between 0.029 and 0.048 mmol g^{-1} dm (**Figure 1C**) and between 0.481 and 0.977 mmol 100 g^{-1} fw, respectively. As already shown for the TEAC and Folin−Ciocalteu assay, the ESR results (**Figure 1C**) reveal the same influence of the genotype on antioxidant activity. The old, traditional cultivars 'Altmärker Braunkohl', 'Halbhoher grüner Krauser', and 'Lerchenzunge' as well as the hybrid cultivar 'Redbor' have antioxidant activities higher than those of all other hybrid cultivars and 'Frostara'. The results of the ESR correlate both strongly and positively with the results of the TEAC assay ($p = 0.810$) and of the Folin−Ciocalteu assay ($p = 0.737$). The ESR is a common, well-known, established method for detecting radicals and their degradation kinetics. Unfortunately, it is seldom used in combination with other assays to determine the overall antioxidant activity. Therefore, a comparison to data of other researchers is lacking. In comparison to the TE values of the $ABTS^{\bullet+}$ scavenging ability (**Figure 1B**), Fremy's salt values (**Figure 1C**) are half that amount. The different reaction time, constitution, and size of the radical could be responsible for this effect.

Influence of Climate (Temperature and Global Radiation) on the Total Phenolic Content. In addition to the genotypic influence, the biosynthesis of phenolic substances can be also influenced by the climatic factors temperature and radiation. Low temperatures are associated with higher concentrations of flavonoids due to the higher quantities of ROS (*6*). At lower temperatures, the mRNA of phenylalanine ammonia lyase and chalcone synthase are enhanced or accumulated in maize seedlings and *Arabidopsis thaliana* (*23*, *24*). Phenolic substances act as shielding components and are responsible for protecting plants from damages caused by radiation, in particular UV-B (*25*). Increasing global radiation induces the synthesis and accumulation of shielding components, such as flavonoids and hydroxycinnamic acids, especially in the epidermis of leaves (*25*). However, none of the literature deals with climatic conditions of low temperature parallel to low radiation for vegetables cultivated in Central Europe in the winter season.

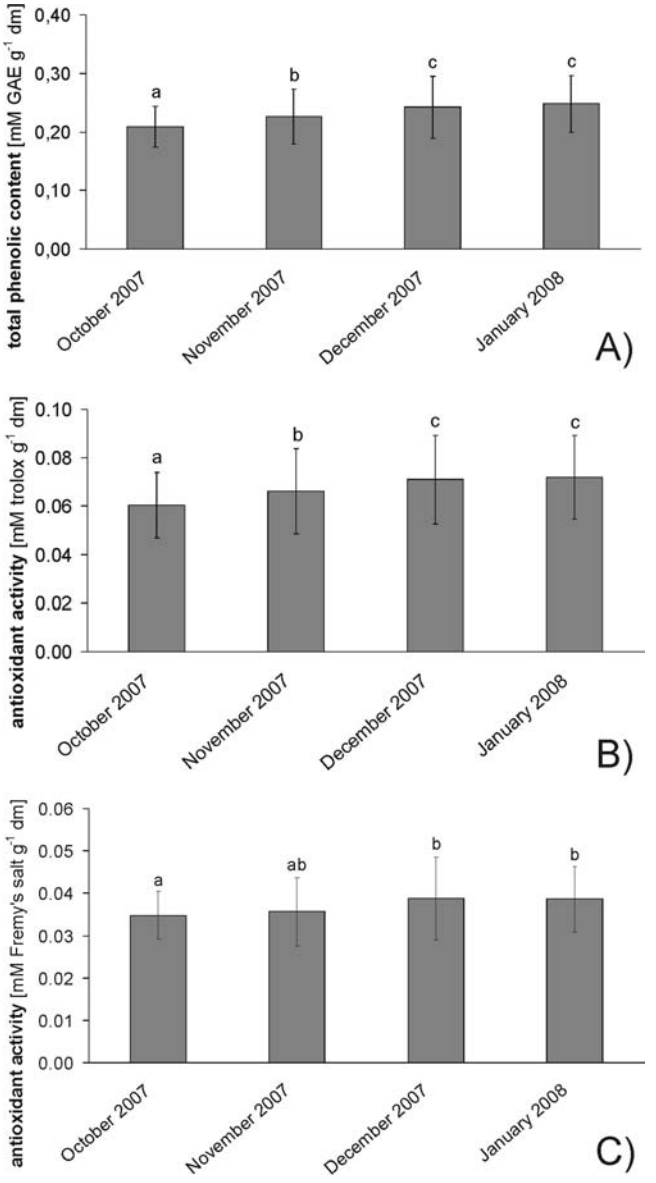

Figure 2. Climatic effect on the total phenolic content (**A**) and the antioxidant activity (measured with TEAC (**B**) and ESR (**C**)) of four harvest times in a 4 week interval between October 2007 and January 2008 irrespective of cultivar. Results were expressed as millmoles of gallic acid equivalents per gram of dry matter (**A**), as millimoles of trolox equivalents per gram of dry matter (**B**), and as millimoles of Fremy's salt per gram of dry matter (**C**). Different letters in each chart indicate significant differences between the cultivars ($p \leq 0.05$ by Tukey's HSD test). Asterisks denote cultivars that have a red leaf color ($n = 3$). Temperature and radiation values were as follows: October 2007, 9.7 °C, 713 μmol m^{-2} s^{-1}; November 2007, 6.0 °C, 285 μmol $m^{-2}s^{-1}$; December 2007, 3.8 °C, 179 μmol m^{-2} s^{-1}; January 2008, 0.3 °C, 172 μmol m^{-2} s^{-1}.

During the field experiment daily mean global radiation and daily mean temperature decreased from 713 to 172 μmol m^{-2} s^{-1} and from 9.7 to 0.3 °C between October 2007 and January 2008, respectively. The decreasing climate factors result in a significant increase of the TPC values irrespective of the cultivar. As seen in **Figure 2A**, a significant enhancement of the TPC was found for

kale harvested in November and December compared to October. Hence, for decreasing temperature ranging from 9.7 to 6.0 to 3.8 °C combined with falling radiation intensities from 713 to 285 to 179 μmol m^{-2} s^{-1} (October to December) the plants react to the climatic-induced changes. The decreasing temperature range from 3.8 to 0.3 °C combined with a decreasing mean radiation level from 179 to 172 μmol m^{-2} s^{-1} (December to January) had no influence on the TPC.

Klimov et al. reported an enhanced TPC for winter wheat, resulting from an enhanced ROS level at low temperatures (*6*). The increase of phenolic compounds could be due to a cold-induced biosynthesis based on the induction of phenylalanin ammonia lyase and chalcone synthase, important enzymes in the flavonoid biosynthesis pathway (*23*, *24*). The investigation of Wildanger et al. also showed an increasing flavonoid concentration during the cultivation of kale at lower temperatures (*7*), whereas Hagen et al. detected a 25−35% decrease of the total phenols and the antioxidant activity in kale that remained 3 and 6 weeks in the field in comparison to kale harvested 1 day after the first night of frost (*8*).

Depending on the cultivar, the preliminary studies of Schmidt et al. showed an increasing quercetin and a decreasing kaempferol concentration with decreasing temperature and radiation (*4*). However, the total flavonol concentration remained unchanged during cultivation at lower temperatures (October to December) (*4*). The increasing TPC of the aforementioned cultivars correlates with the fact that quercetin, as a dihydroxylated flavonol, has a greater reducing ability than the monohydroxylated kaempferol and isorhamnetin (*17*); thus, a higher TPC is measured. Whereas in December and January the concentration of isorhamnetin is enhanced (*4*), the TPC remains unchanged in the present study (**Figure 2A**).

According to the literature, exposure to global radiation should lead to an augmented synthesis of protective compounds, such as flavonoids, which act as ROS scavengers (*26*, *27*). Wildanger et al., Zhang et al., and Romani et al. reported that reduced UV radiation, for example under greenhouse conditions, resulted in lower kaempferol, quercetin, and flavonol glycoside concentrations (*7*, *26*, *28*). For that reason, with regard to the study presented here, a lower TPC was expected in January with a mean global radiation level of only 172 μmol m^{-2} s^{-1} compared to October with 713 μmol m^{-2} s^{-1}. In contrast, the climate conditions caused an increase of the TPC, suggesting that lower temperatures are responsible for the effect due to enhanced ROS concentrations (*6*) and (cold) temperature-induced biosynthesis (*23*, *24*). In addition, the physiological aging of plants is accompanied by an increase in ROS level (*29*), which can also lead to higher TPC values.

Influence of Climate (Temperature and Global Radiation) on the Trolox Equivalent Antioxidant Capacity Assay. In the context of the decreasing mean temperature and mean global radiation, the antioxidant activity, analyzed irrespective of cultivar, increased significantly (**Figure 2B**). A significantly enhanced antioxidant activity was measured when cultivated within the time from October to December with a decreasing mean temperature from 9.7 to 3.8 °C, in combination with a reduced global mean radiation from 713 to 179 μmol m^{-2} s^{-1}. The changes in the measured climatic factors from December to January showed no significant influence on the antioxidant activity.

The influence of the climate conditions on the results of the TEAC assay are consistent with those gained from the actual TPC values and the aglycone concentrations obtained in a preliminary study by Schmidt et al. (*4*). There it was shown that, depending on the genotype, the quercetin concentration increased while the kaempferol concentration decreased from October to December (*4*). Since quercetin is a better antioxidant than kaempferol (*30*), the antioxidant activity presented in this study was increased, whereas from December to January the quercetin concentration of all cultivars except 'Arsis' remained unchanged (*4*) and thus no significant change in the antioxidant behavior was observable (**Figure 2B**).

Table 1. Two-Way Factorial Analysis of Variance To Test for Interactions between the Factors Cultivar, Harvest Time, and Cultivar × Harvest ($p \leq 0.05$). [a]

factor	TPC	TEAC	ESR
cultivar	*	*	*
harvest	*	*	*
cultivar × harvest	ns	*	ns

[a] Legend: *, statistically significant; ns, not significant.

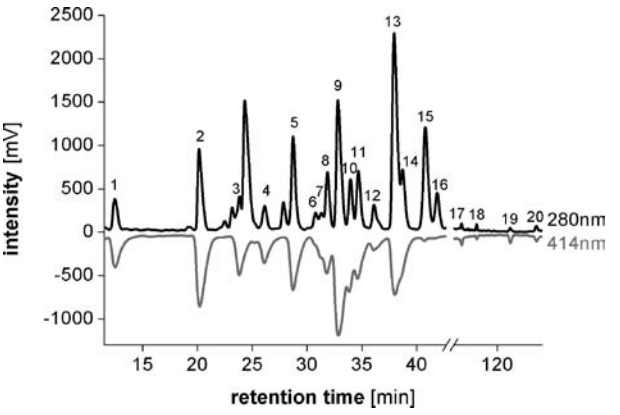

Figure 3. HPLC−UV/vis−online TEAC chromatogram of kale; cv. 'Altmärker Braunkohl' red leaves. (for peak numbers, refer to **Table 2**).

Influence of Climate (Temperature and Global Radiation) on the Antioxidant Activity Measured by Electron Spin Resonance. As mentioned above, ESR was applied to compare the results from the TEAC and Folin−Ciocalteu assays. The influence of the climate conditions on the antioxidant activity analyzed irrespective of cultivar (**Figure 2C**) is similar to those gained from the TEAC and Folin−Ciocalteu assays. In the context of the decreasing mean temperature and mean global radiation, significant changes in the antioxidant activity have been observed (**Figure 2C**). A significantly enhanced antioxidant activity was found for kale harvested in December. The climatic change in the time from December to January showed no significant influence on the antioxidant activity. The ESR results provided evidence that the impact of the decline in temperature could be responsible for this effect.

Interaction between Genotype and Climate (Temperature and Global Radiation). In **Table 1** the analyses of variance reveal a significant influence of the cultivars on the TPC, TEAC, and ESR results irrespective of harvest date and vice versa. The interaction of both statistic factors was observable for the TEAC results but not for the TPC and antioxidant activity by ESR.

Contribution of Single Compounds to the Overall Antioxidant Activity Measured by HPLC−UV/vis−Online TEAC. In the present study, all assays used detect the overall antioxidant activity. To evaluate the contribution of single compounds to the overall antioxidant activity, the TEAC assay was directly coupled to the chromatographic separation (HPLC−online TEAC approach). **Figure 3** shows the HPLC−UV/vis−online TEAC chromatogram of the cultivar 'Altmärker Braunkohl'. The $ABTS^{\bullet+}$ radical used for the postcolumn derivatization is dark green and possesses a maximum absorption at 414 nm. During the reaction of the radical with an antioxidative substance of the HPLC eluate, the signal absorption at 414 nm decreases.

Table 2. Contribution of Flavonoid Glycosides and Hydroxycinnamic Acid Derivatives to the Antioxidant Activity of cv. 'Altmärker Braunkohl' Red[a] by using HPLC-DAD-ESI-MS^n and HPLC−UV/Vis−Online TEAC

compd	R_t (min)	name	total Abs^{280} (%)	total Abs^{414} (%)	TE (mM)	total Abs^{414}/total Abs^{280}
1	12.5	caffeoylquinic acid	2.65	5.83	0.60	2.20
2	20.1	quercetin-3-*O*-sophoroside-7-*O*-glucoside	6.19	11.70	1.20	1.89
3	23.8	quercetin-3-*O*-hydroxyferuloyl-sophoroside-7-*O*-glucoside	2.37	6.03	0.62	2.54
4	26.1	quercetin-3-*O*-caffeoyl-sophoroside-7-*O*-glucoside	2.02	4.62	0.47	2.29
5	28.7	kaempferol-3-*O*-hydroxyferuloyl-sophoroside-7-*O*-glucoside	6.78	8.39	0.86	1.24
6	30.8	kaempferol-3-*O*-hydroxyferuloyl-sophoroside-7-*O*-diglucoside	1.35	0.62	0.06	0.46
7	31.3	quercetin-3-*O*-glucoside-7-*O*-glucoside	1.10	1.71	0.18	1.56
8	31.8	kaempferol-3-*O*-caffeoyl-sophoroside-7-*O*-glucoside	3.98	4.59	0.47	1.15
9	32.8	quercetin-3-*O*-sinapoyl-sophoroside-7-*O*-glucoside quercetin-3-*O*-sophoroside-7-*O*-sinapoyl-diglucoside	10.59	16.67	1.70	1.57
10	33.9	quercetin-3-*O*-triglucoside	3.35	5.35	0.55	1.60
11	34.7	quercetin-3-*O*-feruloyl-sophoroside-7-*O*-glucoside	4.38	6.31	0.64	1.44
12	36.1	quercetin-3-*O*-cumaroyl-sophoroside-7-*O*-glucoside	2.12	2.47	0.25	1.16
13	37.9	kaempferol-3-*O*-sinapoyl-sophoroside-7-*O*-glucoside	16.09	7.41	0.76	0.46
14	38.7	kaempferol-3-*O*-sinapoyl-sophoroside-7-*O*-diglucoside	4.40	4.70	0.48	1.07
15	40.8	kaempferol-3-*O*-feruloyl-sophoroside-7-*O*-glucoside	8.58	0.55	0.06	0.06
16	41.9	kaempferol-3-*O*-triglucoside	2.76	0.28	0.03	0.10
17	98.6	quercetin-3-*O*-disinapoyl-triglucoside-7-*O*-glucoside	1.48	6.19	0.63	4.19
18	107.6	kaempferol-3-*O*-disinapoyl-triglucoside-7-*O*-glucoside	0.89	0.73	0.07	0.82
19	127.7	1,2-disinapoyl-gentiobiose	0.92	4.14	0.42	4.51
20	143.5	1-sinapoyl-2-feruloyl-gentiobiose	1.66	1.72	0.18	1.03

[a] Red describes the leaf color.

Therefore, the peaks from the second detection (negative peaks at 414 nm) can be related to those peaks at 280 nm of the traditional HPLC chromatogram (**Figure 3**). Compounds that are capable of scavenging the $ABTS^{\bullet+}$ radical are numbered (**Figure 3** from 1 to 20). The antioxidant activity of these numbered compounds was calculated as mM TE using trolox at a level of 1 mM as an internal standard. Structural investigation was carried out using UV spectra, parent mass, and fragmentation patterns. The coeluting substances of peak 9 (**Figure 3**), quercetin-3-*O*-sinapoyl-sophoroside-7-*O*-glucoside and quercetin-3-*O*-sophoroside-7-*O*-sinapoyl-diglucoside, are the components with the greatest ability to scavenge the $ABTS^{\bullet+}$ radical and hence contribute the most to the overall antioxidant activity (**Table 2**, column 6). It should be noted that, on the basis of quantity, these compounds have the second largest area under the curve (AUC) value at 280 nm.

Among the antioxidant active compounds (shown in **Table 2**), 10 of them are quercetin, 8 are kaempferol, and 3 are hydroxycinnamic acid derivatives. The compounds having a higher antioxidative activity in comparison to that of trolox were quercetin derivatives (**Table 2**, **2** and **9**). As mentioned above, quercetin (and its derivatives) is more capable of scavenging free radicals than kaempferol due to its catechol structure of the B-ring (*30*, *31*). A second structural feature promoting the antioxidant activity is the kind of conjugated hydroxycinnamic acid of the acylated kale flavonol glycosides. Among the compounds with a high antioxidant activity, five are monoacylated and three are diacylated with sinapic acid (**Table 2**, **9**, **13**, **14**, and **17**−**20**) and three are monoacylated with hydroxyferulic acid (**Table 2**, **3**, **5**, and **6**) and another three with ferulic acid (**Table 2**, **11**, **15**, and **20**) and caffeic acid (**Table 2**, **1**, **4**, and **8**), respectively. One structure shows an acylation with coumaric acid (**Table 2**, peak 12). The glycosidation pattern of 10 of the 19 kale flavonoids identified (**Table 2**) reveals sophorose in position 3-*O* and glucose in position 7-*O* and another three contain sophorose in position 3-*O* and diglucose in position 7-*O*.

When the structures of quercetin and kaempferol are compared, it is assumed that the two structures only differing in their basic structure but being equal in their acylation and/or glycosidation pattern should result in higher TE values for the quercetin derivative. This was found for **10** and **16** as well as for **17** and **18** but not for the pair **3** and **5** as well as **4** and **8**, respectively (**Table 2**). Furthermore, other researchers reported a higher antioxidant capacity for cinnamic acids with a catechol structure compared to cinnamic acids without this structural feature (*30*, *31*). Therefore, in the actual study the flavonol derivatives conjugated with sinapic acid or hydroxyferulic acid should be less antioxidizing than those with caffeic acid (**Table 2**, **4** and **8**). However, this aspect seems not to be always valid: see **3**, **5**, and **8**. According to Rösch et al. the kind of sugar substituent in position 3-*O* has little or no effect on the antioxidant activity of isorhamnetin derivatives (*31*). On comparison of **2** with a TE of 1.2 mM and a diglucoside in position 3-*O* with **7** with a TE of just 0.18 mM and a monoglucoside at 3-*O*, this hypothesis seems not to be true. Interestingly, the conjugation with a monoglucoside at 7-*O* leads to activity higher than for those with a diglucoside, as can be seen for the pair **5** and **6** as well as **13** and **14**, respectively. To summarize, the assumed structure−antioxidant relationships found by many researchers seems not to be applicable in any case. A possible reason could be the different polarities with respect to the solubilities of the compounds, especially for such compounds that are highly glycosidated *and* acylated.

The contribution of a single compound to the overall antioxidant activity depends on its structural characteristics and its amount in the sample. To investigate the substance-specific structure−activity relationship, it is important to erase or equalize the quantity factor. Due to the different polarities with respect to the solubilities of the compounds during the gradient run, quantification on the basis of model substance equivalents (for example "calculated as rutin") was not performed. Instead, the ratio of the areas at 414 and 280 nm was introduced as a parameter for the potential of single compounds to scavenge radicals. Such an approach is independent of the quantity and chemical behavior of the substances.

As is known from the literature, flavonols and their glycosides possess two absorption maxima: band I (350−380 nm) and band II (240−280 nm) corresponding to both the B and the A rings. Conjugation of the hydroxyl groups at position 3, 5, or 4′ causes a band I hypsochromic shift (*32*). Investigations by Day et al. showed that although the band I shift of quercetin monoglucuronides conjugated at different hydroxyl groups can be found,

these glycosides have almost similar absorption properties at 280 nm (*33*). Therefore, in the present study it was assumed that all flavonoid glycosides capable of scavenging $ABTS^{\bullet+}$ show nearly the same absorption properties at 280 nm.

The influence of an acylation with phenolic acids to a possible shifting of band II of the flavonoid glycosides is also negligible. Most of the flavonoid glycosides shown in **Table 2** comprise a mono- or diphenolic acid acylation at the sugar moiety at the 3-*O* or 7-*O* position. It was expected that the aromatic π-electron system of the acylated phenolic acid does not lead to a hypsochromic or bathochromic shift.

As can be seen in **Table 2** (column 7), among the 10 compounds (**Table 2**, **1**−**4**, **7**, **9**, **10**, **17** and **19**) obtaining the highest antioxidant potential, 8 are quercetin glycosides and 2 are hydroxycinnamic acid derivatives. However, the component of **19** in **Figure 3** (see also **Table 2**), 1,2-disinapoyl-gentiobiose, is the most potential antioxidant. It should be noted that a change in the acylation pattern, as for **20** the 1-sinapoyl-2-feruloyl-gentiobiose, leads to an obviously decreased antioxidant activity. As already observed for the results of the antioxidant activity given in mmol TE, the type of conjugated hydroxycinnamic acid seems to have a great influence on the antioxidant potential of a substance. Among the 10 structures with the greatest potential, the first and second are each acylated with two sinapic acids (**Table 2**, **17** and **19**), followed by a conjugation with one hydroxyferulic acid for the third highest potential structure (**Table 2**, **3**) and caffeic acid for the compounds with the fourth and fifth highest potentials (**Table 2**, **1** and **4**), respectively.

The results of the contribution of single compounds to the overall antioxidant activity and the results of the ratio show that the catechol structure of the quercetin derivatives is the reason for a high potential radical scavenging activity. More remarkable is the fact that otherwise, as cited in the literature (*30*, *31*), the flavonoid derivatives esterified with methoxylated hydroxycinnamic acids possess antioxidant capacities higher than those conjugated with hydroxycinnamic acids with catecholic structures (e.g., caffeic acid).

In conclusion, the present study indicates that the most commonly used assays, for example the TEAC assay or the Folin−Ciocalteu assay, are useful to compare the antioxidant activities among samples that were treated in the same manner. However, to investigate the influence and contribution of single compounds to the antioxidant activity of a sample or to investigate the structure−activity relationships of single compounds, coupled HPLC−UV/vis−online TEAC in connection with MS data is a less time-consuming and equally accurate method, instead of isolating and cleaning single compounds for individual analyses.

All investigated assays showed similar genotypic influences on total phenolic contents and antioxidant activities among the old, traditional cultivars and among the hybrid species, except for 'Redbor'. Accordingly, direct and strong correlation between the total phenolic content and the antioxidant activity can be seen. This is the first time that both old, traditional and hybrid cultivars have been compared, and our research is one of the few investigations on cultivars of different colors.

In addition to the genotypic influence, the results reveal the importance of the eco-physiological factors temperature and global radiation. It seems that, due to the enhanced ROS level, low temperature during cultivation has a strong effect on the total phenolic content and antioxidant capacity of kale. To our knowledge there has been no research on decreasing temperature combined with decreasing radiation levels. Therefore, the individual influences of declining temperature and radiation level need further research under well-defined conditions.

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