

Changes in quercetin and kaempferol concentrations during broccoli head ontogeny in three broccoli cultivars*

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Summary

Three broccoli cultivars – spear broccoli ‘Emperor’, crown broccoli ‘Marathon’ and violet broccoli ‘Viola’ – were harvested during head ontogeny from start of head development until over maturity stage (five stages) in three different years. The aglycones quercetin and kaempferol were analysed at optimised conditions of acid hydrolysis by HPLC. Heads of over maturity stage had the highest contents of quercetin and kaempferol. However, the genotype fundamentally determined the quantity and course of the increase in flavonols. Mini broccoli, as a new trend to market vegetables, has lower content of flavonols than the commercial stage, which indicates a reduction in health potentials. Harvesting broccoli heads of over maturity stage should be used as raw material, e.g. for the design of new functional foods.

Introduction

Polyphenols are powerful antioxidants *in vitro*, scavenging a range of reactive species and binding transition metals in forms less active in catalysing free radical damage (HALLIWELL, 2006). An epidemiological study in the Netherlands (the Zytphen study) suggests an inverse correlation between the incidence of coronary heart disease and stroke, and the dietary intake of flavonoids, especially quercetin (HERTOG et al., 1993). Quercetin, the main representative of the flavonol class, found in high concentrations in onions, apples, red wine, broccoli, tea and *Ginkgo biloba*, influences some carcinogenic markers and has minor effects on plasma antioxidant biomarkers *in vivo*, although some studies failed to find this effect (WILLIAMSON and MANACH, 2005).

Flavonoids have in common a basic C₆-C₃-C₆ skeleton structure, consisting of two aromatic rings and a heterocycle containing one oxygen atom. Flavonoids usually occur in plants as glycosides substituted with various sugars – mostly glucose, but also galactose, rhamnose, and other sugars. In broccoli, five different glycosides of quercetin and kaempferol were identified, whereby quercetin 3-O-sophoroside and kaempferol 3-O-sophoroside were the main flavonoid glycosides (PRICE et al., 1998).

Phenolic compounds may be affected by numerous preharvest factors. VALLEJO et al. (2003a) found that, besides genetic variation, extreme agronomic and environmental conditions (late seasons and rich sulphur fertilisation) induce different stress situations on broccoli, enhancing the phenolic content, such as flavonoids and hydroxycinnamoyl derivatives. In contrast, organic farming and water stress decreased the overall production of phenolics in broccoli, such as glycosylated flavonoids and hydroxycinnamic esters (ROBBINS et al., 2005). The developmental stage at which a vegetable is harvested can also influence its phytochemicals.

In fruit vegetables, such as pepper, it was found that immature green peppers had a very high flavonoid content, while green, immature red and ripe red peppers demonstrated a 4- to 5-fold reduction (MARIN et al., 2004). Otherwise, the flavonoid concentration of peppers de-

creased, increased or remained constant, while maturity was dependent on the cultivar (HOWARD et al., 2000). In leafy vegetables such as baby spinach, it was found that younger baby spinach leaves had the highest concentration of total flavonoids (BERGQUIST et al., 2005). Furthermore, flavonol glycosides decreased with increasing age of pear leaves (ANDREOTTI et al., 2006). Besides the consumption of fully developed broccoli heads by consumers, broccoli can also be used as a raw material and as ready-to-eat mini broccoli – a new market product (SCHREINER et al., 2006). VALLEJO et al. (2003b) found that the total flavonoid concentration and caffeoyl-quinic acid derivatives, sinapic and ferulic acid derivatives in broccoli increased from the first development stage until over maturity. They investigated broccoli grown in a relatively warm climate in Spain, with relatively low head diameters at the commercial maturity stage of 13-14 cm, in comparison with 21-23 cm head diameters from Northern Europe.

The aims of this study were (1) to determine the influence of head ontogeny on quercetin and kaempferol concentration in broccoli under the relatively cool climatic conditions of Northern Europe, and (2) to clarify whether similar trends can be observed between both flavonoids – quercetin and kaempferol – and between broccoli cultivars. Samples were taken at five stages of head development for three broccoli cultivars in three different years. Furthermore, based on the method of HERTOG et al. (1992), conditions of acid hydrolysis were optimised for the determination of the aglycones quercetin and kaempferol in broccoli.

Materials and methods

Plant material

Three broccoli cultivars were used – ‘Emperor’ (spear type, heavy green), ‘Marathon’ (crown type, grey-green) and ‘Viola’ (violet type) – and experiments were performed in autumn in three different years: experiment I in 2002, planting date: July 25, harvest date: September 13 to November 4, with 17.4°C daily mean temperature and 246 $\mu\text{mol m}^{-2} \text{s}^{-1}$ daily mean sum of photosynthetic photon flux density (PPFD); experiment II in 2003, planting date: July 22, harvest date: September 9 to October 13, with 17.5°C daily mean temperature and 263 $\mu\text{mol m}^{-2} \text{s}^{-1}$ daily mean sum of PPFD; and experiment III in 2004, planting date: July 22, harvest date: September 10 to October 26, with 16.6°C daily mean temperature and 289 $\mu\text{mol m}^{-2} \text{s}^{-1}$ daily mean sum of PPFD.

The experiments were set up with 3-fold field repetition (sandy soil, pH 6.8) with a plot size of 20 m², containing 5.5 plants m². Cultivation (fertilisation, irrigation and plant protection) was performed according to ‘Integrated Production’ guidelines (WINKHOFF, 1992). Protection nets, which reduced light by 20%, were used to minimise chemical plant applications.

All cultivars were planted at the same time and harvested at different times once the respective head size had been reached. Since the broccoli types differed with regard to their typical head size at harvest, measurement of head diameters was performed by an additional determination of the physiological stage at harvest. According to the BBCH scale (uniform coding of phenologically similar growth stages

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of plant species) by MEIER (1997), we classified the harvested heads in five development stages: 1, start of head development; 2 and 3, mini broccoli; 4, fully developed heads (usual commercial stage); and 5, over maturity stage (first individual flowers visible). The head diameter was measured following the head surface curvature (Tab. 1).

Tab. 1: Head surface curvature diameter (cm) at harvest at five head development stages. Values represent the mean of three experiments and tree replications.

Convar.	Head development stages				
	1	2	3	4	5
Emperor	6.40±0.78	12.35±0.75	16.04±0.71	21.77±0.95	23.32±2.01
Marathon	6.62±0.74	12.12±0.75	16.13±0.82	22.34±1.74	26.47±2.92
Viola	6.10±0.58	11.49±0.96	15.95±0.96	21.90±1.61	23.33±3.08

Flavonoid analysis

A mixed sample of florets (bud and second-order branching) of each replication was taken, consisting of 10 heads at development stage 1 and 5 heads each at stages 2 to 5. Only the florets were used for analyses, since these are the broccoli parts that are commonly consumed (SCHONHOF and KRUMBEIN, 1996). The material was immediately deep-frozen (-40°C), then freeze-dried and finely ground. Flavonols were determined as their aglycones after acid hydrolyses. Unless otherwise stated, extracts of broccoli samples were prepared in the following way: 40 ml of 62.5% aqueous methanol was added to 0.5 g of the freeze-dried broccoli sample. 10 ml of 8 M HCl was added to this extract. Thus, the extraction solution consisted of 1.6 M HCl in 50% aqueous methanol (v/v). After refluxing at 90°C for 2h, the extract was allowed to cool, was adjusted to 100 ml with 50% methanol, and sonicated for 5 min. The extract was then filtered through a 0.45 µm filter for HPLC analyses.

The flavonoid composition and concentration were determined using a series 1100 HPLC (Agilent, Waldbronn, Germany) equipped with a diode array detection system. A Prodigy column ODS(3) (250 x 4.6 mm, 5 µm, 100Å) (Phenomenex, Aschaffenburg, Germany) was used with a security guard C18 (4 x 3.0 mm) at a temperature of 25°C. Solvent A was water + 0.1% TFA (trifluoroacetic acid) + 2% THF (tetrahydrofuran); solvent B was acetonitrile. The following gradient was used: 30 - 35% B (5 min), 35 - 39% B (12 min), 39 - 90% B (5 min), 90% B isocratic (2 min), 90 - 30% B (5 min), 30% B isocratic (5 min). The chromatogramme were monitored at 270 nm with a flow rate of 1 ml min⁻¹. Contents were quantitatively determined by calibration curves of the related pure standards (quercetin, Fluka, Buchs, Switzerland; kaempferol, Carl Roth GmbH, Karlsruhe, Germany). The results were converted to 100 g fresh matter (fm).

Quercetin and kaempferol were identified by HPLC-ESI-MS², using Agilent 1100 series (ion trap) in the negative ionisation mode. Nitrogen was used as dry gas (12 l min⁻¹, 350°C) and nebuliser gas (40 psi). To compare the results with the HPLC-DAD, the same column material with a shorter length (150 x 4.6 mm, 5 µm, 100 Å) was used. TFA in solvent A was replaced by 0.5% (v/v) acetic acid, THF in solvent A was omitted and the same water/acetonitril gradient and a flow rate of 0.6 ml min⁻¹ were used. Quercetin and kaempferol were identified from the deprotonated molecular ions [M - H]⁻ with m/z 301 and 285, respectively. Furthermore, in MS² quercetin and kaempferol showed a characteristic mass fragment of ring A at m/z 151, which is typical for several flavonoids (MARCH et al., 2006).

Statistical analysis

The results were evaluated using analysis of variance, and least significant differences were calculated using Tukey's Honest-Significant-

Difference (HSD) test (5% significance level). The data from different planting dates (three years) were pooled, resulting in nine replications per treatment.

Results and discussion

Optimised sample preparation and HPLC method of flavonoids in broccoli

The extraction efficiency of 0.5 g of a freeze-dried broccoli sample with 50% and 70% aqueous methanol was comparable, but higher than the use of 30% aqueous methanol (data not shown). Fifty percent aqueous methanol was therefore used for all analyses. The hydrolysis of all glycosides to aglycons offers a practical method for the quantitative determination of flavonoids in foods (HERTOG et al., 1992). The authors found that the completeness of hydrolysis for the determination of quercetin, kaempferol, myricetin, luteolin and apigenin investigated in cranberry, onion, leek, lettuce, endive and celery depended on both the reaction period and acid concentration, and that the matrix composition was an important factor determining the rate of hydrolysis. Therefore, the influence of hydrolysis times up to 6h (0.5, 1, 2, 3, 4, 6h) and three different hydrochlorid acid concentrations (1.2 M HCl, 1.6 M HCl, 2 M HCl) on a peak area of quercetin and kaempferol in the same freeze-dried broccoli material was investigated. Figs. 1 and 2 show the results for quercetin and kaempferol, respectively, in broccoli florets. For both flavonols, the highest concentration in broccoli was reached when 1.6 M HCl was used, with a hydrolysis time of 2h. A hydrolysis time of 4 h and more led to a loss of flavonols. This corresponds to studies performed by HERTOG et al. (1992) who proposed a hydrolysis with 1.2 M HCl in boiling 50% aqueous methanol with a reaction period not exceeding 2h and, eventually, a second analysis under identical conditions with a reaction period of 4h for the quantification of kaempferol glucosides. We found that 1.2 M HCl needs longer hydrolysis times of 3 h for the determination of quercetin and kaempferol in broccoli, and is therefore ineffective. 1.6 M HCl and a hydrolysis time of 2 h were therefore used for all analyses.

Using a Prodigy ODS column and optimised water (TFE, THF)/acetonitrile gradient (see material and method), a good separation of quercetin and kaempferol from other unidentified compounds could be attained within 15 minutes. The recovery of quercetin and kaempferol in broccoli florets spiked with 50% of the original level was 93% for both flavonols. Coefficients of variation ranged from 4.3% to 3.5% for quercetin and kaempferol, respectively.

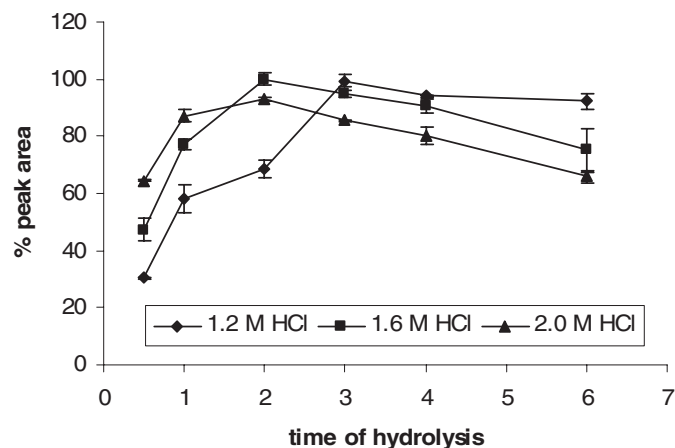


Fig. 1: Influence of hydrolysis time and acid concentration on quercetin. Results are expressed as a percentage of the highest peak area found in broccoli florets. The values represent the mean of two replicates ± standard deviation.

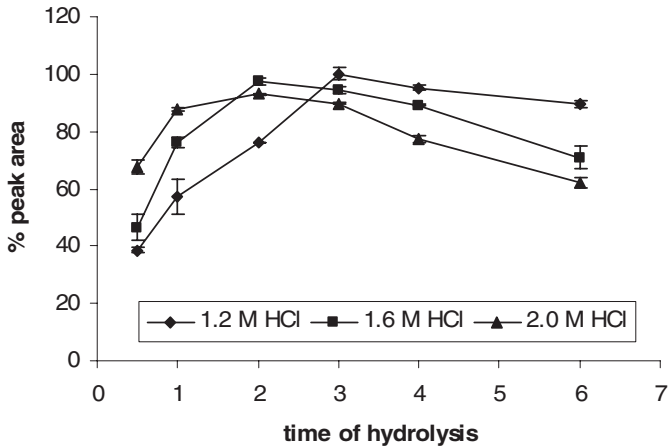


Fig. 2: Influence of hydrolysis time and acid concentration on kaempferol. Results are expressed as a percentage of the highest peak area found in broccoli florets. The values represent the mean of two replicates ± standard deviation.

Effect of head ontogeny

The changes of quercetin and kaempferol with head ontogeny showed the same trend in the three experiments, enabling the values to be averaged over three years. Heads of over maturity stage (stage 5, first individual flowers visible) had the highest contents of quercetin and kaempferol (Figs. 3-5). VALEJO et al. (2003) also found that the total flavonoid concentration in broccoli increased from the first development stage until over maturity stage, although the head weights investigated by VALLEJO et al. (2003b) were far lower – with an average of 260 g for fully developed heads compared to 352 g for ‘Emperor’ and ‘Viola’, as well as 396 g for ‘Marathon’ in our experiments. However, the genotype fundamentally determined the quantity and course of the increase in flavonols. The highest concentration of quercetin and kaempferol was found in the cultivar Viola in the over maturity stage, with 6.8 and 9.9 mg (100 g fm)⁻¹, respectively. The concentration of quercetin increased 3-fold in heads of over maturity stage compared to the beginning of the head formation (stage 1) in ‘Emperor’, 5-fold in ‘Marathon’ and 14-fold in ‘Viola’. Kaempferol correspondingly increased 5-fold in ‘Emperor’, 8-fold in ‘Marathon’ and 17-fold in ‘Viola’. This means that kaempferol increased more intensively than quercetin during head development. While a continuous increase in quercetin and kaempferol took place in ‘Viola’ in all examined head stages of development, both flavonols increased in ‘Marathon’ in fully developed heads (stage 4), and once again in heads of over maturity stage (stage 5). Similar results were obtained found for kaempferol in ‘Emperor’. The concentration of quercetin remained unchanged in ‘Emperor’, even up to the fully developed heads. Changes in the ratios of quercetin to kaempferol were determined during head ontogeny. The proportional composition of about 45% quercetin and 55% käämpferol remained relatively constant from the head formation up to fully developed heads in ‘Viola’; the proportion of kaempferol then increased up to 59% in heads of over maturity stage. The proportion of kaempferol increased during head ontogeny by 17% and 15% in ‘Emperor’ and ‘Marathon’, respectively. Differences in the quantitative formation of quercetin and kaempferol during head formation may be caused by the various enzymes involved in each flavonol’s synthesis from the flavanone naringenin. The hydroxylation of naringenin to dihydrokaempferol is catalysed by the enzyme flavanone 3-hydroxylase (FHT), and further conversion to kaempferol is catalysed by the enzyme flavonol synthase (FLS) (FORKMANN and HELLER, 1989). In contrast, besides these enzymes the biosynthesis of quercetin also needs the enzyme flavonoid 3’-hydroxylase (F3’H), which uses the flavanone naringenin and/or the

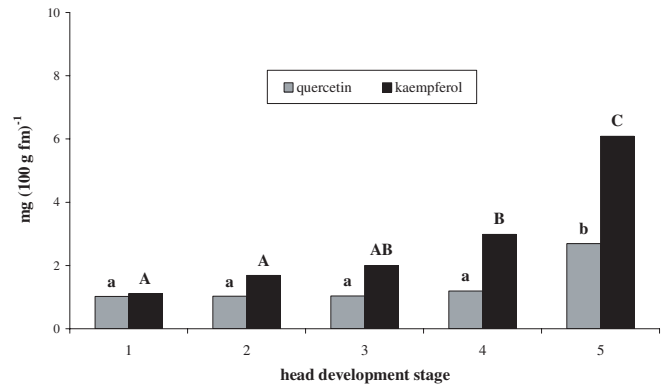


Fig. 3: Quercetin and kaempferol concentrations of broccoli florets with advanced head development during five development stages in cv Emperor. Values represent the mean of three experiments and three replications. Different letters indicate significant differences during head development for each flavonol (p<0.05 by Tukey’s HSD test).

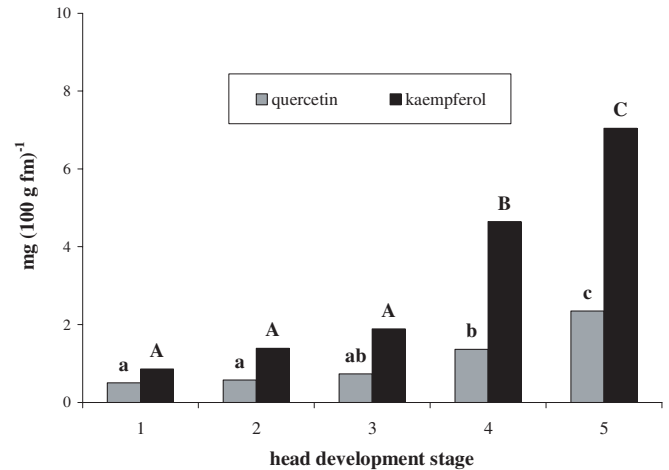


Fig. 4: Quercetin and kaempferol concentrations of broccoli florets with advanced head development during five development stages in cv Marathon. Values represent the mean of three experiments and three replications. Different letters indicate significant differences during head development for each flavonol (p<0.05 by Tukey’s HSD test).

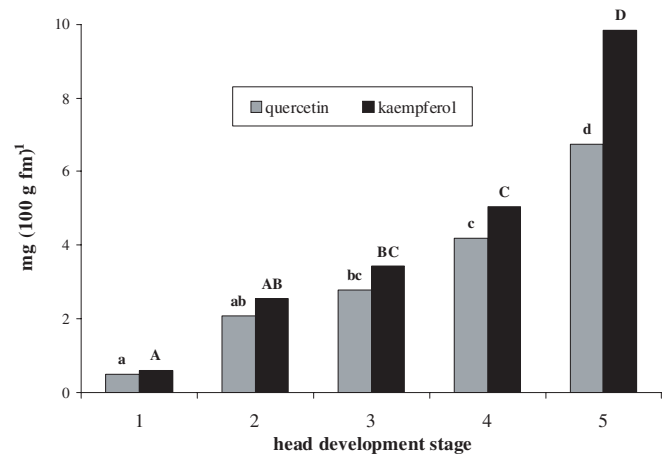


Fig. 5: Quercetin and kaempferol concentrations of broccoli florets with advanced head development during five development stages in cv Viola. Values represent the mean of three experiments and three replications. Different letters indicate significant differences during head development for each flavonol (p<0.05 by Tukey’s HSD test).

dihydroflavonol dihydrokaempferol as a substrate for quercetin biosynthesis and/or hydroxylates kaempferol directly to quercetin (FORKMANN and HELLER, 1989). The activity of F3'H was found in the microsomal fraction; the reaction required molecular oxygen and NADPH (FORKMANN and HELLER, 1989).

According to different applications, different head development stages of broccoli should be produced and offered to guarantee a high level of the desired flavonols quercetin and kaempferol. Dependent on the genotype, fully developed broccoli heads eaten by the consumers already contain relatively high concentrations of flavonols. Out of the investigated cultivars, 'Viola' had the highest flavonol concentration. Consumers should not only eat green broccoli, but could also benefit especially from consuming the violet variety, although, purple broccoli does not seem to correspond to consumers' aesthetic expectations (SCHONHOF et al., 2004). Mini broccoli as a new trend to market vegetables has lower contents of flavonols than the commercial stage, which indicates a reduction in health potentials. Harvesting broccoli heads of over maturity stage resulted in maximum flavonol levels. For this reason, these stages should be used as raw material, e.g. for the design of new functional foods.

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