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# Free and bound phenolic compounds in leaves of pak choi (*Brassica campestris* L. ssp. *chinensis* var. *communis*) and Chinese leaf mustard (*Brassica juncea* Coss)

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ABSTRACT

Eleven pak choi cultivars and two leaf mustard cultivars grown under field conditions in China were investigated for the free polyphenol content in their outer and inner leaves, as well as in their leaf blades and leaf stalks. In most cases, there were no significant differences between the hydroxycinnamic acid derivative and flavonoid derivative contents in the outer and inner leaves for the 13 cultivars. However, the contents of blades and stalks differed: hydroxycinnamic acids and flavonoids were present in greater amounts in the leaf blade than in the leaf stalk. Trace or small amounts of flavonoids were detected in the pak choi and leaf mustard stalks. Additionally, the bound phenolic contents of two pak choi cultivars and two leaf mustard cultivars were investigated. The concentrations of cell wall-bound phenolic compounds were higher in the leaf blade than in the leaf stalk under field conditions in China. These compounds represent only a minor portion of the total phenolic contents (flavonoids and hydroxycinnamic acids) in leaf stalks (0.81–1.18%) and leaf blades (0.05–0.08%) from fresh plant material. The storage of plant samples from four Chinese cabbage cultivars resulted, in most cases, in an increase of phenolic content, within six days, at 4 °C and 20 °C. The increase might have been triggered by post-harvest plant stresses, which stimulate the biosynthesis of polyphenols.



## 1. Introduction

The biosynthesis and concentration of polyphenols in plants depends on climatic conditions, harvest seasons, agricultural and environmental factors, and post-harvest treatments. The polyphenol contents may differ between cultivars, as well as within the individual plant (e.g. blades vs. stalks), as shown for pak choi grown under greenhouse conditions in a recent study (Harbaum et al., 2007). Bahorun, Luximon-Ramma, Crozier, and Aruoma (2004) reported on the contents of different flavonoid aglycones in hydrolyzed vegetable extracts. Of all the vegetables studied, Chinese cabbage possessed the highest content with approximately 4.5 mg/g dm (dry material, total content determined by HPLC); broccoli was found to contain approximately 2 mg/g dm. Low concentrations of the compounds were reported for white cabbages and cauliflower (approximately 0.5–0.6 mg/g dm). Sakakibara, Honda, Nakagawa, Ashida, and Kanazawa (2003) found pak choi to contain the highest levels, with a total content of approximately 77–222 μmol/100 g fresh material (fm); broccoli contained approximately 32 μmol/100 g fm, cabbage approximately 21 μmol/100 g fm, and cauliflower 8 μmol/100 g fm (only hydroxycinnamic acids were present; no flavonoids were detected in cauliflower). Vallejo, Tomas-Barberan, and Garcia-Viguera (2003) detected flavonoid and hydroxycinnamic acid levels of approximately 1–10 mg/g dm and 0.5–2 mg/g dm, respectively, in broccoli inflorescences. Price, Casuscelli, Colquhoun, and Rhodes (1998) determined flavonoid derivatives in broccoli florets at levels of 2.1 mg/g dm. Tolonen et al. (2002) reported very low kaempferol contents (9 μg/g dm) in white cabbages; kaempferol was the only flavonoid found. Kim, Padilla-Zakour, and Griffiths (2004) detected about 0.1–0.8 mg/g fm of quercetin and kaempferol aglycone content in green cabbages. The phenolic content in tronchuda cabbage amounted to approximately 0.036–0.172 mg/g dm in internal leaves and 0.027–1.95 mg/g dm in external leaves (Ferreres et al., 2005; Sousa et al., 2005), as well as approximately 1.4 mg/g dm in internal leaves and approximately 13 mg/g dm in external leaves (Ferreres et al., 2006). Miean and Mohamed (2001) detected approximately 0.15–0.22 mg/g of total flavonoid content (aglycones by HPLC) for broccoli, cauliflower, cabbage, and Chinese cabbage. These values were all of the same magnitude.

The main polyphenols identified in Chinese cabbages are acylated mono-, di-, tri-, and tetraglucosides of kaempferol, as well as esters of hydroxycinnamic acids with malic acid, glycosides,

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and quinic acid (Harbaum et al., 2007; Harbaum, Hubbermann, Zhu, & Schwarz, 2008; Rochfort, Imsic, Jones, Trenerry, & Tomkins, 2006). It is well known that the structural properties of polyphenols affect the rate and extent of their absorption in the small intestine and colon of humans, as well as the formation and occurrence of metabolites in plasma. Glycoside and ester linkages in flavonoids influence the absorption rate and site in the human intestine, due to specific sugar transporters in the small intestine or the activity of the microorganism flora (esterase and glucosidase activity) in the colon (Scalbert & Williamson, 2000). Phenolic compounds are present as free, as well as cell wall-bound compounds, in plants. It has been known since the 1980s that covalently linked phenolic acids play a role in connection with diets, based on fibre-rich plants in the transformation of the intestinal microflora in guinea pig (Knehans & O'Dell, 1980). Dietary fibre plays an important role in the human diet: in addition to exerting generally beneficial effects, it is associated with a reduction in cancer and coronary heart disease rates (Anonymous, 2001; Jacobs, Slavin, & Marquart, 1995; Truswell, 2002). Bound phenolic compounds significantly affect the biodegradability and fermentation rate of dietary fibre in the human colon. The degree of degradation by microorganisms in the human colon is dependent on their esterification level with monomeric compounds and the resulting cross-linkages between cell wall components. Hopkins et al. (2003) showed a reduction in the fermentation rate of ferulic acid cross-linked arabinoxylans in the colon compared to non-cross-linked arabinoxylans. The absorption, physiological functions, and bioavailability of cell wall-bound phenolic compounds differ from those of free phenolic compounds. Bound compounds (such as ferulic acid) are only released by enzymatic activity (esterase) (Andreasen, Kroon, Williamson, & Garcia-Conesa, 2001a; Kroon, Faulds, Ryden, Robertson, & Williamson, 1997), and the release may be influenced by their bonding to the dietary fibre. The food matrix, e.g. of cereals, influences the bioavailability of ferulic acid, and the limitation of their absorption may provide information about their association with dietary fibre. The quantity of bound ferulic acid excreted in urine was approximately 90–95% lower than that of free ferulic acid, but the levels were high in fecal excretion (Adam et al., 2002). According to Buchanan, Wallace, Fry, and Eastwood (1996), monomeric compounds are absorbed, whereas dimeric compounds accumulate as enzyme-resistant fragments in the gastrointestinal tract. By contrast, Andreasen et al. (2001a) observed the intestinal microbial release of esterified dimers (such as diferulic acids) from cereals, as well as further absorption via the gastrointestinal tract.

There is growing interest in the beneficial properties of dietary phenolic acid antioxidants. Moderate beer consumption may result in decreased diseases such as cancer or cardiovascular disease due to the antioxidative activity of bound phenolic compounds in barley (Szwajgier, Pielecki, & Targonski, 2005a). Several studies have shown the enhanced antioxidative potential of bound phenolic compounds compared to non-bound phenolics (Liyana-Pathirana & Shahidi, 2006; Ohta, Yamasaki, Egashira, & Sanada, 1994), as well as the antioxidative potential of dimeric and monomeric compounds (Andreasen, Landbo, Christensen, Hansen, & Meyer, 2001b; Garcia-Conesa, Wilson, Plump, Ralph, & Williamson, 1999). For this reason, more information about the ratio of bound to free phenolic compounds in plants of commonly consumed fruits and vegetables is necessary to gauge the importance of these compounds in the human diet. However, there are only limited data about the contents of both free and bound polyphenols in various plant cultivars, particularly in dicotyledons.

Polyphenols (flavonoids and hydroxycinnamic acids) were detected in pak choi plants that had been cultivated in greenhouses (Rochfort et al., 2006; Harbaum et al., 2007) and differentiated into leaf blades and leaf stalks (Harbaum et al., 2007), but not according to different plant parts (outer vs. inner leaves). The aim of this work is to identify the various concentrations of free and cell wall-bound phenolics in Chinese cabbages (leaf mustard and pak choi plants) cultivated under field conditions in China and Germany. The influence of post-harvest treatments, such as storage, on phenolic content will be taken into account.

## 2. Materials and methods

### 2.1. Chemicals

Acetonitrile (HPLC grade, Fisher Scientific), *p*-coumaric acid (Sigma), ethylacetate (Carl Roth GmbH), ferulic acid (Carl Roth GmbH), formic acid (Carl Roth GmbH), conc. HCl (Carl Roth GmbH), *p*-hydroxybenzaldehyde (Carl Roth GmbH), kaempferol-3-*O*-hydroxyferuloyldiglucoside-7-*O*-glucoside [isolated compound according to Harbaum et al. (2007)], metaphosphoric acid (Fluka), methanol (HPLC grade, Fisher Scientific), oxalic acid dihydrate (Carl Roth GmbH), sinapic acid (Carl Roth GmbH), sodium dodecylsulphate (Carl Roth GmbH), sodium hydroxide (Carl Roth GmbH), sodium metabisulphite (Carl Roth GmbH), sodium sulphate (Carl Roth GmbH) trifluoroacetic acid (Carl Roth GmbH), vanillic acid (Carl Roth GmbH), and vanillin (Carl Roth GmbH) were utilized.

### 2.2. Plant material for the determination of free phenolic compounds

The plants of 11 pak choi cultivars and two leaf mustard cultivars were grown under field conditions in China in triplicate ($n = 3$). The outer and inner leaves of the 10 week-old plants were harvested, separated into leaf blades and stalks, freeze-dried, and stored at ambient temperature under dry conditions prior to further analysis.

### 2.3. Plant material for the storage assay

Two pak choi cultivars (cv. Hangzhou You Dong Er and cv. Shanghai Qing) and two leaf mustard cultivars (cv. Xue Li Hong and cv. Bao Bao Qing Cai) were grown under field conditions in Germany. The plants were harvested after five weeks, at which point each plant was packed in sheets (perforated: 30 μm diameter) and stored at 4 °C in a refrigerator and at 20 °C. After 0, 2, 4, 6, and 8 days, samples were prepared in triplicate ($n = 3$) for each cultivar. The plants were freeze-dried and stored at ambient temperature under dry conditions prior to further analysis.

### 2.4. Extraction of free phenolic compounds

The extraction of free phenolic compounds was carried out according to Harbaum et al. (2007). Freeze-dried and ground plant material (ca. 0.3 g) was extracted four times (4 ml, 2 ml, 2 ml, and 2 ml) with acidic aqueous methanol (containing 1% metaphosphoric acid and 0.5% oxalic acid dihydrate) facilitated by ultrasonication (1 min for each step) and centrifuged. The collected supernatants of each extraction step were made up to 10 ml and filtered. The filtrate was stored at −20 °C prior to further analysis.

### 2.5. HPLC analysis of free phenolic compounds

Quantitative HPLC analysis of polyphenols in the obtained extracts was carried out on a HP1100 HPLC (Agilent Tech., Waldbronn, Germany) equipped with a diode array detector according to Harbaum et al. (2007). Separation was carried out on a 250 × 4 mm i.d., 5 μm, RP-18 Nucleodur column with an 8 × 4 mm Nucleodur guard column at 20 °C. Eluent A consisted of 0.15% trifluoroacetic acid in water and eluent B of 100% acetonitrile at a flow rate of 0.9 ml/min. Gradient elution started with 10% B for

9 min, reaching 12.5% B after 13 min and 14% B at 19 min. It remained at 14% B until 35 min, reaching 26% B after 60 min, 70% B after 75 min, and 10% B after 77 min and until 80 min. Compounds were detected and quantified by UV absorption at 330 nm. Injection volume was set to 25 μl. Kaempferol-3-*O*-hydroxyferuloyldiglucoside-7-*O*-glucoside, which was obtained by the isolation procedure as presented by Harbaum et al. (2007), was used as a reference compound for quantification (detection limit 0.002 mg/ml). The quantification of hydroxycinnamic acids was carried out relative to the external standard sinapic acid (detection limit 0.001 mg/ml) and further calculations were performed utilizing the molecular weight of the main identified hydroxycinnamic acid compound sinapoylmalate: molecular weight factor mwf = M[sinapoylmalate = 340]/M[sinapic acid = 224].

### 2.6. Plant material for the determination of bound phenolic compounds

Two pak choi cultivars (cv. Hangzhou You Dong Er and cv. Shanghai Qing) and two leaf mustard cultivars (cv. Xue Li Hong and cv. Bao Bao Qing Cai) were grown under field conditions in China. The 10 week-old plants were harvested and separated into leaf blades and stalks. Cell wall isolation was carried out in duplicate for each plant sample ($n = 2$).

### 2.7. Isolation of cell wall material

The isolation of cell wall material was carried out with a method described by Beveridge, Loubert, and Harrison (2000) for *Brassica* vegetables and with some modifications of the procedure presented for young cabbage leaves (Selvendran & Ryden, 1990). Fresh plant material was first reduced to small pieces and then comminuted in a crushing machine. Two-fold 1.5% SDS solution, containing 5 mM sodium metabisulphite (approximately 600 ml), was added to the plant material (approximately 300 g) and disrupted by Ultra Turrax treatment (12,000 $min^{-1}$) for 5 min. The suspension was then stirred for 15 min and filtered through a nylon mesh. The residue was washed again with 0.5% SDS solution containing 3 mM sodium metabisulphite (3 times). Afterwards, the residue was rinsed three times with 3 mM sodium metabisulphite solution and three times with distilled water. The white residue was washed with ethanol, three times, to remove any free phenolic compounds or other interfering substances. The freeze-dried residue was ball milled and kept dry at an ambient temperature in the dark prior to further analysis.

### 2.8. Hydrolysis reaction of cell wall material and extraction

Approximately 300 mg of the isolated and dried cell wall material were hydrolyzed with 25 ml 1 M NaOH for 96 h under $N_2$ in the dark to release the bound phenolic compounds (saponification). The suspension was filtered and the filtrate was further acidified to pH < 2 with conc. HCl. The acidified solution was extracted three times with ethyl acetate and the combined and dried (with $Na_2SO_4$) organic phases were evaporated to dryness under $N_2$. The residue was dissolved in 500 μl of 25% aqueous methanol and analyzed by HPLC–DAD. Each cell wall hydrolysis reaction was performed in triplicate ($m = 3$).

### 2.9. HPLC analysis of bound phenolic compounds

The HPLC analysis of bound phenolic compounds was carried out on an Agilent HPLC (HP1100, Agilent Tech. Waldbronn, Germany) equipped with a diode array detector. Separation was carried out on a 250 × 4 mm i.d., 5 μm, RP-18 Nucleodur column with an 8 × 4 mm Nucleodur guard column at 20 °C. Eluent A consisted of 0.1% formic acid in water (HPLC-grade) and eluent B of 100% acetonitrile at a flow rate of 0.7 ml/min. Gradient elution was started with 0% B and reached 10% B after 7 min, 19% B after 20 min, 22% B after 25 min, 23% B after 26 min, 24% B after 35 min, 50% B after 50 min, and 100% B after 64 min. Compounds were identified with standard compounds and quantified at 280 nm for hydroxybenzoic acids and hydroxybenzaldehydes and at 330 nm for hydroxycinnamic acids. Injection volume was set to 100 μl. Calibration was carried out with the analogous standard compounds for each identified phenolic compound. *cis*-Ferulic acid was calculated by the standard curve of *trans*-ferulic acid. The detection limits of standard compounds ranged from 0.2 to 0.5 μg/ml.

### 2.10. Statistical analysis

Standard deviations and significant differences were calculated by SPSS 15.0 (one-way ANOVA, $p < 0.05$, Bonferroni).

## 3. Results and discussion

### 3.1. Quantification of free phenolic compounds

Eleven cultivars of pak choi and two cultivars of Chinese leaf mustard were grown in China, differentiated into outer and inner leaves, as well as into leaf blades and leaf stalks, and investigated for free phenolic compounds (flavonoids and hydroxycinnamic acids). The detected contents are presented in Table 1. The flavonoid content was highest for the pak choi cv. Ai Kang Qing (38.7 mg/g dm), in the leaf blades of the inner leaves, and lowest in the cv. Huang Xin Cai (15.0 mg/g dm). Cv. Shanghai Qing had the highest concentrations (33.1 mg/g dm), in the leaf blades of the outer leaves, of all the pak choi cultivars; the Hei You Bai Cai cultivar had the lowest (14.2 mg/g dm). The flavonoid contents of the leaf mustard and pak choi cultivars were found to be of the same magnitude. Flavonoids were detected in trace or low amounts in the leaf stalks in this study (Table 1), but were not detected at all in any of the 11 pak choi cultivars grown under greenhouse conditions in Germany (Harbaum et al., 2007).

The cv. Hangzou You Dong Er had the highest hydroxycinnamic acid content (7.05 mg/g dm) in the outer leaves and leaf blades of all pak choi cultivars; the highest content for the inner leaves and leaf blades was detected in the cv. Shanghai Qing (7.71 mg/g dm). Both leaf mustard cultivars had lower hydroxycinnamic acid contents in the leaf blades of the outer and inner leaves than had pak choi cultivars.

In most cases, the hydroxycinnamic acid contents in outer and inner leaves were not significantly different, except in cv. Suzhou Qing and cv. Huang Xin Cai, whose leaf stalk values were significantly lower in the inner leaf. Significantly higher flavonoid contents in the inner leaf blades were only present in the cultivars Ai Jiao Huang and Si Yue Man, which led to the overall assumption that there are no marked differences in phenolic content in the outer and inner leaves. For the most part, the observed differences between the outer and inner leaves were less pronounced than those in tronchuda cabbages according to the literature, which showed ten-fold higher concentrations in the tronchuda's outer leaves (approximately 1–10 mg/g) than in the inner leaves (approximately 0.1–1 mg/g) (Ferreres et al., 2005, 2006; Sousa et al., 2005). This may be related to the cabbage habitus: Some cabbages, e.g. white cabbages, have a compact structure (head cabbages). Only their external leaves are irradiated by sunlight, which might account for the very low polyphenol content in their inner leaves. By contrast, pak choi is a leafy cabbage, i.e. less compact, which allows more of its leaves to receive light and produce great quantities of polyphenols in the plant (Lois, 1994).

**Table 1**
Phenolic content in eleven pak choi cultivars and two leaf mustard cultivars cultivated under field conditions in China, separated into leaf blade and leaf stalk as well as outer and inner leaf ($n = 3$)

| Chinese cabbage | Cultivar | Plant part | Outer leaf | | | Inner leaf | | |
|---|---|---|---|---|---|---|---|---|
| | | | Blade/stalk ratio[a] | Hydr.cinn.acids (mg/g dm)[b] | Flavonoids (mg/g dm)[c] | Blade/stalk ratio[a] | Hydr.cinn.acids (mg/g dm)[b] | Flavonoids (mg/g dm)[c] |
| Pak choi | Ai Kang Qing | Blade | 0.47 | 6.76 ± 0.40 | 29.6 ± 4.53 | 0.55 | 7.27 ± 0.39 | 38.7 ± 3.80 |
| | | Stalk | | 1.17 ± 0.14 | 0.65 ± 0.15 | | 1.12 ± 0.12 | 0.70 ± 0.30 |
| | Lu Xiu | Blade | 1.27 | 6.27 ± 0.57 | 24.9 ± 3.91 | 0.36 | 7.11 ± 1.00 | 33.8 ± 5.38 |
| | | Stalk | | 1.19 ± 0.31 | Traces | | 0.95 ± 0.12 | 0.40 ± 0.01 |
| | Suzhou Qing | Blade | 0.91 | 4.98 ± 0.69 | 14.9 ± 1.45 | 0.83 | 5.53 ± 1.53 | 20.5 ± 4.26 |
| | | Stalk | | 1.12 ± 0.15 | Traces | | 0.78 ± 0.11* | Traces |
| | Shanghai Qing | Blade | 0.74 | 6.70 ± 0.50 | 33.1 ± 1.86 | 0.92 | 7.71 ± 1.33 | 19.8 ± 12.56 |
| | | Stalk | | 1.83 ± 0.31 | 1.63 ± 0.50 | | 1.25 ± 0.50 | Traces |
| | Nanjing Zhong Gan Bai | Blade | 0.61 | 6.74 ± 1.22 | 21.3 ± 2.20 | 1.06 | 5.16 ± 0.86 | 18.4 ± 3.10 |
| | | Stalk | | 1.22 ± 0.20 | Traces | | 0.93 ± 0.15 | Traces |
| | Hei You Bai Cai | Blade | 0.71 | 5.97 ± 0.38 | 14.2 ± 3.19 | 0.95 | 5.58 ± 0.07 | 15.9 ± 2.97 |
| | | Stalk | | 1.71 ± 0.12 | 0.73 ± 0.08 | | 1.46 ± 0.21 | Traces |
| | Al Jiao Huang | Blade | 0.86 | 5.79 ± 0.57 | 23.3 ± 1.85 | 0.85 | 5.85 ± 0.23 | 28.3 ± 0.86* |
| | | Stalk | | 0.95 ± 0.11 | Traces | | 0.72 ± 0.34 | Traces |
| | Si Yue Man | Blade | 0.58 | 6.85 ± 0.50 | 22.6 ± 2.43 | 0.59 | 7.37 ± 0.47 | 31.8 ± 5.42* |
| | | Stalk | | 1.35 ± 0.14 | Traces | | 1.11 ± 0.26 | Traces |
| | Si Ji Xiao Bai Cai | Blade | 1.14 | 6.26 ± 0.84 | 22.3 ± 2.91 | 1.20 | 6.09 ± 0.37 | 20.5 ± 2.65 |
| | | Stalk | | 1.36 ± 0.15 | 0.54 ± 0.12 | | 1.13 ± 0.37 | Traces |
| | Huang Xin Cai | Blade | 1.10 | 7.03 ± 0.35 | 16.1 ± 7.80 | 1.27 | 6.70 ± 0.31 | 15.0 ± 2.00 |
| | | Stalk | | 1.85 ± 0.26 | 0.78 ± 0.13 | | 0.78 ± 0.40* | Traces |
| | Hangzhou You Dong Er | Blade | 0.43 | 7.05 ± 0.87 | 28.9 ± 0.56 | 0.39 | 6.06 ± 0.67 | 26.8 ± 3.74 |
| | | Stalk | | 1.37 ± 0.43 | Traces | | 0.74 ± 0.24 | Traces |
| | Mean value[d] | Blade | | 6.40 ± 0.63 | 22.8 ± 6.15 | | 6.40 ± 0.87 | 24.5 ± 7.84 |
| | | Stalk | | 1.37 ± 0.30 | nc[e] | | 1.00 ± 0.24 | nc[e] |
| Leaf mustard | Bao Bao Qing Cai | Blade | 0.75 | 5.07 ± 0.02 | 27.9 ± 1.93 | 0.62 | 3.53 ± 1.85 | 26.3 ± 10.64 |
| | | Stalk | | 0.99 ± 0.46 | 1.75 ± 0.26 | | 0.85 ± 0.49 | 1.46 ± 0.56 |
| | Xue Li Hong | Blade | 0.78 | 4.40 ± 1.44 | 25.9 ± 0.64 | 0.95 | 4.42 ± 0.44 | 23.5 ± 6.41 |
| | | Stalk | | 1.57 ± 0.22 | 1.34 ± 0.35 | | 1.79 ± 0.14 | 1.52 ± 0.13 |
| | Mean value[d] | Blade | | 4.74 ± 0.47 | 26.9 ± 1.44 | | 3.97 ± 0.63 | 24.9 ± 2.00 |
| | | Stalk | | 1.28 ± 0.41 | 1.55 ± 0.29 | | 1.32 ± 0.66 | 1.49 ± 0.04 |

[a] Calculated on fresh weight basis.
[b] Expressed as sinapoylmalate equivalents [calibrated by sinapic acid and further calculated by the molecular weight factor 'mwf' = 340/224, as presented by Harbaum et al. (2007)]; significant differences are indicated by the (*) symbol.
[c] Expressed as kaempferol-3-*O*-hydroxyferuloyldiglucoside-7-*O*-glucoside equivalents; significant differences are indicated by the (*) symbol.
[d] Mean value for the contents of all cultivars combined.
[e] nc = not calculated, due to predominantly trace amounts of flavonoids in stalks.

Phenolic concentrations, particularly flavonoids, were found to be very high under field conditions in China compared to other cabbages: approximately 14–39 mg/g of dm for flavonoids and 3.5–7.7 mg/g for hydroxycinnamic acids in the leaf blades. The phenolic contents of other cabbage cultivars are lower; broccoli has approximately 1–10 mg/g dm (Price et al., 1998; Vallejo et al., 2003), while white cabbages, green cabbages, and tronchuda cabbages exhibit approximately 0.01–10 mg/g dm (Ferreres et al., 2005; Kim et al., 2004; Sousa et al., 2005; Tolonen et al., 2002). These findings also led to the assumption that the cabbage habitus plays a significant role in polyphenolic biosynthesis, which is influenced by light supply (Lois, 1994). Overall, the phenolic contents detected in pak choi are realistic, due to the natural calibration standard and main compound kaempferol-3-*O*-hydroxyferuloyl-diglucoside-7-*O*-glucoside present in pak choi, as shown in a recent study (Harbaum et al., 2007). However, the purchased standard compounds (e.g. aglycones or simple monoglycosides) for calibration for the determination of polyphenols by HPLC often underestimate the real phenolic content of plants.

Flavonoid content was much higher than hydroxycinnamic acid content in the leaf blades of all the investigated cultivars of pak choi and leaf mustard. The main flavonoids are kaempferol derivatives (mono-, di-, triglucosides), as shown for pak choi and Chinese leaf mustard in recent studies by HPLC–ESI–$MS^n$ analysis (Harbaum et al., 2007, 2008). Isorhamnetin derivatives were also detected, but no quercetin derivatives were found, even though the plants were grown under field conditions in China [high sunlight supply, compared to greenhouses (Harbaum et al., 2007)].

Overall, kaempferol derivatives, the main flavonoids in cabbages, were also presented for tronchuda (Ferreres et al., 2005, 2006), green (Kim et al., 2004), and white cabbages (Nielsen, Norbaek, & Olsen, 1998; Nielsen, Olsen, & Petersen, 1993); the derivatives are glucosylated and acylated. Glucosylated and acylated flavonoid derivatives are mostly identified in *Brassica* vegetables, as shown for cauliflower, turnip tops, white cabbage, tronchuda cabbage, pak choi and Chinese leaf mustard (Ferreres et al., 2005; Harbaum et al., 2007, 2008; Llorach, Gil-Izquierdo, Ferreres, & Tomas-Barberan, 2003; Nielsen et al., 1993, 1998; Rochfort et al., 2006; Romani, Vignolini, Isolani, Ieri, & Heimler, 2006).

The hydroxycinnamic acid content was always higher in the leaf blade than the stalk, for all cultivars. Gentiobiose derivatives, which were identified in previous studies in pak choi (Harbaum et al., 2007), were mainly detected in the inner leaves (individual content not shown) in all cultivars of pak choi and leaf mustard. Ferreres et al. (2006) also identified ferulic acid and sinapic acid derivatives of gentiobiose, predominantly in the inner leaves of tronchuda cabbage. The main hydroxycinnamic acids were malate derivatives of sinapic acid, ferulic acid, hydroxyferulic acid, and caffeic acid, as presented for pak choi cultivars in previous studies (Harbaum et al., 2007). These compounds were also detected in other members of the Brassicacea family, e.g. *Brassica rapa* (Abdel-Farid, Kim, Choi, & Verpoorte, 2007; Liang et al., 2006).

Overall, the qualitative analysis including HPLC chromatograms and the detailed identification procedure by HPLC–ESI–$MS^n$ of free polyphenols in pak choi and Chinese leaf mustard cultivars were presented in recent works (Harbaum et al., 2007, 2008).

Additionally, the contents for the whole plants of the cultivars Hangzhou You Dong Er, Shanghai Qing, Xue Li Hong, and Bao Bao Qing Cai, grown under field conditions in Germany, are presented in Table 2 (day 0). Flavonoid content was of the same magnitude for plants grown in China and Germany when the entire plant was considered. However, hydroxycinnamic acid content was lower in the plants grown in Germany than in those cultivated in China, particularly for cv. Shanghai Qing and cv. Xue Li Hong.

The optimum temperature for the cultivation of Chinese cabbages, such as pak choi and leaf mustard, is approximately 20 °C. A daytime length of approximately 10–14 h results in flowering (Keller, 1986). Chinese cabbage requires mild climatic conditions (20 °C), and cultivation is limited to the spring and autumn seasons in China. The Chinese cabbages in this study were cultivated in autumn (September–November), when temperatures were about 20 °C in the Zhejiang region of China; the plants were grown for ten weeks. In Northern Germany, the plants were cultivated in August and September due to the 20 °C temperatures in those months; however, the longer daytime length in the summer resulted in flowering, so the plants were harvested after five weeks. The shorter cultivation period also resulted in shorter exposure to sunlight during plant development, and the formation of flowers might account for the lower hydroxycinnamic acid content in the German plants (Table 2). In cooler seasons (winter, autumn, spring), the cultivation of pak choi is carried out under greenhouse conditions in Germany due to the shorter days and controlled temperatures, as presented in previous studies (Harbaum et al., 2007). The differences in light exposure between greenhouse cultivation [max. mean light intensity measured at noon: 250 $\mu mol\ m^{-2}\ s^{-1}$ (Harbaum et al., 2007)] and field cultivation [Germany: maximum mean light intensity at noon in the present study: 750 $\mu mol\ m^{-2}\ s^{-1}$; maximum value in the sun: 1700 $\mu mol\ m^{-2}\ s^{-1}$] might be responsible for the differences in phenolic content under field conditions and greenhouse conditions: In the present study, the phenolic contents of pak choi, cultivated under field conditions in China (separated into blades and stalks) and Germany (whole leaves), were approximately two- to three-fold higher [mean values in China in leaf blade: hydroxycinnamic acids 6.4 mg/g; flavonoids: 23–25 mg/g dm (Table 1)] than in pak choi grown in Germany under greenhouse conditions [mean values in leaf blade: hydroxycinnamic acids 3.7 mg/g; flavonoids 8 mg/g (Harbaum et al., 2007)], or than those presented in other studies on pak choi (Rochfort et al., 2006). It is well known that the synthesis of polyphenols is affected by UV radiation and that the biosynthesis of flavonoids in plants is induced by UV (Li, Ou Lee, Raba, Amundson, & Last, 1993; Lois, 1994).

### 3.2. Influence of storage on phenolic content

The results of the storage experiments are presented in Table 2. Hydroxycinnamic acid levels showed an increase until day six, and then remained constant or decreased in most cases until day eight at 4 °C and 20 °C. In most cases, the flavonoid content increased at 4 °C and 20 °C until day two, four, or six, and then decreased until day eight, particularly for the cv. Xue Li Hong. In most cases, the hydroxycinnamic acid content at day six was approximately two-fold higher than the initial content at day 0 at 4 °C and 20 °C; this represents a large increase in the first days of storage of Chinese cabbages. Gil, Ferreres, and Tomas-Barberan (1998) also reported an increasing content of polyphenols for material stored in MAP (modified atmosphere packaging) as well as in air-stored Swiss chard (8 days, 6 °C). This might be an indication of further biosynthesis of polyphenols for plant protection in the first days after harvest (Starzynska, Leja, & Mareczek, 2003), presumably triggered as a reaction to stress in the plants. It is well known that polyphenols such as flavonoids play an important role in plant defence mechanisms and against environmental stress (Dixon & Paiva, 1995). However, Gil, Ferreres, and Tomas-Barberan (1999) reported a constant total flavonoid content for fresh-cut spinach during storage at 10 °C for seven days. Furthermore, it has been reported that longer storage times resulted in decreased phenolic content, e.g. for flavonol glucosides in onions (Price, Bacon, & Rhodes, 1997). In the present study, storage at 20 °C, in particular, resulted in rapid yellowing and floppy leaves (senescence by day two), which resulted in an undesirable appearance and sensory quality for consumers. Storage at lower temperatures might be the only

**Table 2**
Influence of storage on free phenolic content in different cultivars of Chinese cabbage cultivated in Germany under field conditions ($n = 3$)

| Cultivar | Storage time (d) | 4 °C[a] | | 20 °C[a] | |
|---|---|---|---|---|---|
| | | Hydr.cinn.acids (mg/g dm)[b] | Flavonoids (mg/g dm)[c] | Hydr.cinn.acids (mg/g dm)[b] | Flavonoids (mg/g dm)[c] |
| Hangzhou You Dong Er | 0 | 2.29 ± 0.40* | 10.3 ± 1.86 | 2.29 ± 0.40 | 10.3 ± 1.86 |
| | 2 | 2.11 ± 0.60* | 9.29 ± 1.85 | 3.42 ± 0.52 | 9.97 ± 1.24 |
| | 4 | 3.34 ± 0.44*,** | 10.4 ± 0.91 | 2.52 ± 0.09 | 8.72 ± 1.10 |
| | 6 | 4.49 ± 0.19** | 14.9 ± 3.20 | 3.23 ± 1.16 | 7.22 ± 2.63 |
| | 8 | 2.84 ± 0.72* | 11.4 ± 2.67 | 3.00 ± 0.57 | 7.97 ± 4.48 |
| Shanghai Qing | 0 | 1.35 ± 0.41 | 8.50 ± 0.82 | 1.35 ± 0.41* | 8.50 ± 0.82 |
| | 2 | 2.79 ± 0.85 | 11.0 ± 0.98 | 2.10 ± 0.45* | 9.00 ± 2.84 |
| | 4 | 2.67 ± 0.71 | 12.0 ± 4.41 | 3.15 ± 0.70*,** | 10.1 ± 2.95 |
| | 6 | 3.14 ± 0.17 | 12.0 ± 2.04 | 4.11 ± 0.73** | 7.04 ± 1.22 |
| | 8 | 3.05 ± 0.65 | 11.2 ± 3.65 | 2.44 ± 1.07*,** | 4.83 ± 3.77 |
| Xue Li Hong | 0 | 0.90 ± 0.15* | 8.93 ± 1.01* | 0.90 ± 0.15 | 8.93 ± 1.01* |
| | 2 | 1.99[d] | 13.6[d] | 2.75 ± 0.08* | 16.4 ± 0.37** |
| | 4 | 2.71 ± 0.15** | 17.0 ± 2.88** | 2.79 ± 0.37* | 15.4 ± 4.23*,** |
| | 6 | 3.33[d] | 17.3[d] | 3.37[d] | 16.5[d] |
| | 8 | 1.90 ± 0.32*,** | 13.9 ± 1.72*,** | 1.73 ± 0.51 | 8.82 ± 2.78* |
| Bao Bao Qing Cai | 0 | 1.97 ± 0.45 | 18.0 ± 0.42 | 1.97 ± 0.45 | 18.0 ± 0.42 |
| | 2 | 2.00 ± 0.22 | 21.7 ± 1.95 | 2.09 ± 0.22 | 19.5 ± 3.42 |
| | 4 | 2.16 ± 0.13 | 19.8 ± 1.79 | 2.97 ± 0.77 | 19.4 ± 1.93 |
| | 6 | 2.26 ± 0.21 | 20.7 ± 2.25 | 2.89 ± 0.70 | 19.0 ± 0.93 |
| | 8 | 1.67 ± 0.25 | 17.0 ± 1.32 | 3.33 ± 0.50 | 13.2 ± 1.03* |

[a] Storage temperature.
[b] Expressed as sinapoylmalate equivalents [calibrated by sinapic acid and further calculated by the molecular weight factor mwf = 340/224, as presented by Harbaum et al. (2007)]; significant differences are indicated by the (*, **) symbols.
[c] Expressed as kaempferol-3-*O*-hydroxyferuloyldiglucoside-7-O-glucoside equivalents; significant differences are indicated by the (*, **) symbols.
[d] $n = 2$.

practicable method for retaining freshness. By contrast, no distinct increase in polyphenolic content was detected after two days of plant withering, as presented in previous studies (Harbaum et al., 2008), which may be explained by the differences in the water content during the storage conditions (withering resulted in the reducing of the plant moisture content by 50% whereas storage in sheets prevented the reduction of moisture), and therefore, differences in enzymatic activities are assumed.

### 3.3. Quantification of cell wall-bound phenolic compounds

The bound phenolic content was investigated for two pak choi cultivars (cv. Hangzhou You Dong Er and cv. Shanghai Qing) and two leaf mustard cultivars (cv. Xue Li Hong and cv. Bao Bao Qing Cai). Table 3 presents the bound phenolic content after a 96 h hydrolysis time with 1 M NaOH in the leaf blades, as well as the leaf stalks, of the four cultivars. The detected bound phenolics were vanillic acid, *p*-hydroxybenzaldehyde, vanillin, *p*-coumaric acid, sinapic acid, *trans*-ferulic acid, and *cis*-ferulic acid. The total content was two-fold higher in the leaf blade than in the leaf stalk for both pak choi cultivars, whereas there were no significant differences with respect to the different plant parts for the leaf mustard cv. Xue Li Hong. The cv. Bao Bao Qing Cai exhibited 1.5 times higher content (significant) in the leaf blade than in the leaf stalk. The total content ranged from 96.6 to 143 μg/g dm in the leaf blade and 70.2–89.5 μg/g in the leaf stalk. These results are of the same

**Table 3**
Bound phenolic content in different cultivars of Chinese cabbage cultivated in China under field conditions

| Phenolic compound | cv. Hangzhou You Dong Er | | cv. Shanghai Qing | |
|---|---|---|---|---|
| | Blade (ug/g cell wall)[a] | Stalk (ug/g cell wall)[a] | Blade (ug/g cell wall)[a] | Stalk (ug/g cell wall)[a] |
| Vanillic acid | 9.1 ± 1.1 | 10.4 ± 1.5* | 11.8 ± 3.2 | 9.5 ± 1.7* |
| *p*-Hydroxybenzaldehyde | 12.6 ± 1.1 | 3.7 ± 0.7* | 12.7 ± 1.5 | 4.4 ± 1.6* |
| Vanillin | 54.0 ± 3.3 | 31.6 ± 8.1* | 46.5 ± 2.6 | 35.4 ± 12.6* |
| *p*-Coumaric acid | 5.2 ± 0.4 | 1.6 ± 0.3* | 5.9 ± 0.2 | 2.1 ± 0.9* |
| Sinapic acid | 4.5 ± 1.3 | 2.1 ± 0.5* | 3.8 ± 0.5 | 3.8 ± 0.4* |
| *trans*-Ferulic acid | 48.4 ± 2.2 | 18.3 ± 4.1* | 51.1 ± 8.7 | 18.1 ± 4.4* |
| *cis*-Ferulic acid | 8.9 ± 0.9 | 2.5 ± 0.9* | 9.0 ± 0.6 | 3.5 ± 0.7* |
| Total content[b] | 143 ± 6.8 | 70.2 ± 13.6* | 141 ± 11.6 | 76.7 ± 26.8* |
| | cv. Xue Li Hong | | cv. Bao Bao Qing Cai | |
| Vanillic acid | 7.8 ± 1.0 | 11.6 ± 1.0* | 8.4 ± 0.5 | 8.0 ± 1.1* |
| *p*-Hydroxybenzaldehyde | 7.6 ± 1.2 | 7.6 ± 0.9* | 11.6 ± 0.7 | 8.1 ± 2.3* |
| Vanillin | 40.5 ± 11.3 | 57.0 ± 6.4* | 53.5 ± 4.6 | 46.8 ± 11.9 |
| *p*-Coumaric acid | 3.9 ± 1.2 | 2.9 ± 0.9 | 5.6 ± 0.6 | 4.8 ± 1.1 |
| Sinapic acid | 2.8 ± 0.4 | 2.5 ± 0.2* | 6.7 ± 0.9 | 6.3 ± 1.3* |
| *trans*-Ferulic acid | 30.2 ± 5.1 | 7.0 ± 0.7* | 35.5 ± 4.7 | 12.0 ± 1.2* |
| *cis*-Ferulic acid | 3.9 ± 0.9 | 1.0 ± 0.2* | 5.4 ± 0.8 | 2.0 ± 0.2* |
| Total content[b] | 96.6 ± 20.4 | 89.5 ± 8.1 | 127 ± 12.4 | 88.0 ± 17.5* |

[a] Content in dried cell wall ($n = 2$, $m = 3$); significant differences are indicated by the (*) symbol.
[b] Total content of all individual compounds combined.

magnitude as those found for broccoli, which displayed a total of approximately 90 μg/g cell wall after a 24 h hydrolysis time (1 M NaOH) (Beveridge et al., 2000). However, other vegetables (such as carrots) possess higher levels, i.e. total contents of approximately 1.5 mg/g cell wall (Beveridge et al., 2000) or approximately 300–600 μg/g cell wall (Ng, Parr, Ingham, Rigby, & Waldron, 1998).

*trans*-Ferulic acid content was significantly higher in the leaf blades than in the stalks for all investigated cultivars. This was also observable for other compounds, such as *p*-hydroxybenzaldehyde, *p*-coumaric acid, and *cis*-ferulic acid, in both pak choi cultivars. *cis*-Ferulic acid content was significantly higher in the leaf blade than in the stalk of both leaf mustard cultivars. However, no differences were found for the compound vanillic acid between the blades and stalks for three of the cultivars (Hangzhou You Dong Er, Shanghai Qing, and Bao Bao Qing Cai), and no tendency was observable for the main compound vanillin. Renard, Wende, and Booth (1999) detected lower cell wall-bound phenolic content in quinoa stalks than in blades.

In most cases, the standard deviations were predominantly 5–15% of the individual content, but some higher standard deviations result from variations in the cell wall material ($n = 2$; e.g. *trans*-ferulic acid content in the leaf blade of cv. Shanghai Qing: mean values of two isolated cell walls amounted to 43.2 ± 1.9 μg/g and 58.9 ± 1.7 μg/g).

### 3.4. Ratio between free and bound phenolic compounds

The free and bound phenolic content in fresh plant material was calculated, based on the dry weight (for free phenolic compounds) and yield of isolated cell walls from fresh plant material (for bound phenolic compounds). The data are presented in Table 4. The yield of isolated cell walls based on fresh plant material is higher in leaf blades (1.69–1.87%) than in leaf stalks (0.82–1.25%) [according to the literature, the dietary fibre content in pak choi is approximately 2.5% (based on fresh plant material) (Vollendorf & Marlett, 1993)]. The yield of broccoli cell wall material is reported to be 0.87% in the literature and is of the same magnitude as the cell wall yield of pak choi leaf stalks (Beveridge et al., 2000). The yield of cell wall material found in pak choi leaf blades is of the same magnitude as e.g. that detected in asparagus (1.76–2.12% of fresh weight) (Rodriguez-Arcos, Smith, & Waldron, 2002).

The total bound phenolic content, differentiated into the leaf blades and leaf stalks of fresh plant material, differs from the total content of the cell wall material. The cv. Hangzhou You Dong Er showed two-fold higher content in leaf blades than in stalks from cell wall material (Table 3) and five-fold higher content in the leaf blades than in stalks from fresh plant material (Table 4). The total bound phenolic content was in the range of 1.6–2.6 μg/g fm (fresh material) in leaf blades and 0.6–1 μg/g fm in leaf stalks for the four cultivars. The free hydroxycinnamic acid content ranged from 384 to 860 μg/g fm in the leaf blade and from 31 to 80 μg/g fm in the leaf stalk. Therefore, bound phenolic compounds account for approximately 0.31–0.55% in blades and 1.16–2.79% in stalks of the total hydroxycinnamic acid content, as well as 0.05–0.08% in blades and 0.81–1.18% in stalks, with respect to total phenolic content (hydroxycinnamic acids and flavonoids combined). Bound phenolics account for only a minor fraction of the overall content (bound and free phenolic compounds) of polyphenols in Chinese cabbages. These differences are also observable for the *Brassica* vegetable broccoli: The flavonoid content is 278 μg/g fresh weight, as described by Price et al. (1998). Bound phenolic compounds were estimated to be approximately 0.8 μg/g fresh material, based on a cell wall proportion of 0.87% fresh material (Beveridge et al., 2000), and therefore, bound phenolic compounds account for approximately 0.3% of the total phenol content in broccoli. By comparison, in carrots (*Daucus carota* L.), hydroxycinnamic acid deriv-

**Table 4**
Proportions of bound and free phenolic compounds in different cultivars of Chinese cabbages cultivated in China under field conditions

| Cultivar | Plant part | Bound phenolics | | Free phenolics[b] | | | | Proportion of bound phenolics of total contents | |
|---|---|---|---|---|---|---|---|---|---|
| | | Cell wall (% fm)[a] | Total content (ug/g fm) | Dry weight (% fm) | Hca[c] (ug/g fm) | Flavonoids[d] (ug/g fm) | Total content[e] (ug/g fm) | Hca[f](%) | Hca + flavonoids[g](%) |
| Hangzhou You Dong Er | Blade | 1.82 | 2.6 | 12.8 | 839 | 3567 | 4406 | 0.31 | 0.06 |
| | Stalk | 0.82 | 0.6 | 4.65 | 49 | Traces | 49 | 1.16 | 1.16 |
| Shanghai Qing | Blade | 1.87 | 2.6 | 11.9 | 860 | 3159 | 4019 | 0.31 | 0.07 |
| | Stalk | 1.25 | 1.0 | 5.22 | 80 | Traces | 80 | 1.18 | 1.18 |
| Xue Li Hong | Blade | 1.70 | 1.6 | 11.7 | 517 | 2893 | 3409 | 0.31 | 0.05 |
| | Stalk | 1.09 | 1.0 | 3.90 | 65 | 56 | 121 | 1.49 | 0.81 |
| Bao Bao Qing Cai | Blade | 1.69 | 2.1 | 8.92 | 384 | 2420 | 2804 | 0.55 | 0.08 |
| | Stalk | 1.01 | 0.9 | 3.36 | 31 | 54 | 85 | 2.79 | 1.04 |

[a] fm = fresh material.
[b] Mean values of the contents in outer and inner leaves (Table 1) calculated on fresh material.
[c] hca = hydroxycinnamic acids; expressed as sinapoylmalate equivalents [calibrated by sinapic acid and further calculated by the molecular weight factor mwf = 340/224 as presented by Harbaum et al. (2007)].
[d] Expressed as kaempferol-3-*O*-hydroxyferuloyldiglucoside-7-*O*-glucoside equivalents.
[e] Total contents of free hydroxycinnamic acids and flavonoids combined.
[f] Proportion of the total contents of hydroxycinnamic acids (free and bound combined).
[g] Proportion of the total contents of hydroxycinnamic acids (free and bound) and flavonoids combined.

atives amount to 0.76–2.79 μmol/g of fresh material (Sakakibara et al., 2003). Bound phenolics account for approximately 2.5% of the total phenolic content. This estimation is based on 40 μg/g of cell wall-bound phenols in fresh material, which contains 2.59% of cell wall material (Beveridge et al., 2000). These values for pak choi, broccoli, and carrots may give an impression of the magnitude of bound phenolic compounds compared to free phenolic compounds in different vegetables. By contrast, for beer, it was reported that bound phenolic compound contents were 4–6-fold higher than the corresponding free phenolic compounds (Szwajgier, Pielecki, & Targonski, 2005b). Mattila, Pihlava, and Hellström (2005) reported either no or low amounts of free phenolic compounds in commercial grain products, but bound phenolic content was high (in the order of mg/g), and Gallardo, Jimenez, and Garcia-Conesa (2006) also detected higher amounts of bound phenolics than free phenolics in grains. It was shown that the absorption of bound phenolics was lower than that of free phenolic compounds and that these accumulate in the intestine for later excretion via feces (Adam et al., 2002).

## 4. Conclusion

The bound fraction of total phenolic compounds is low in Chinese cabbage. These minor compounds in plants may have a beneficial effect on human health, e.g. by protecting the gastrointestinal tract against certain cancers but, in contrast to the great concentrations of free phenolic compounds, these bound phenolics in Chinese *Brassica* vegetables may play only a minor role in human nutrition and *Brassica* might be a less important source of bound phenolics than cereals and grains. On the other hand, the degradation of non-cross-linked Chinese cabbage cell walls by xylanase and esterase activity in the colon may be easier and result in cell wall fragments, which may create positive health benefits.

Furthermore, the increasing levels of polyphenols in the plant from post-harvest treatments (storage) open up possibilities for increased phenolic content in vegetables and foods.

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