Effects of cultivation strategies on phytochemicals and sensory properties of cabbage (*Brassica oleracea* L. var. *capitata* L.) and curly kale (*Brassica oleracea* L. var. *sabellica* L.)

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June 2014
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Acknowledgement

With the submission of this thesis I partially fulfill the requirements for obtaining the title of Doctor of Philosophy from the Department of Food Science, Aarhus University, Denmark. The work behind this thesis has been conducted throughout the last four years (including 10 months of maternity leave) with great support from different people, who I would like to thank.

Firstly my supervisor, Hanne L. Kristensen, with whom I spent a lot of time discussing experimental plans, results, and manuscripts, but also life in general as a scientist working at the university. Thank you for great collaboration and engagement. I particularly enjoyed our deep scientific discussions. My co-supervisor Kai Grevsen, whose door was always open, especially when I had questions related to statistical issues. The same thanks go to Karen Koefoed Petersen, despite her not being my official co-supervisor.

I am grateful for the great work carried out in the greenhouse, fields, laboratory, and office with help from skilled technicians Astrid Bergmann, Knud Erik Petersen, Jens Barfod, Jens Elkjær, Marta Kristensen, Ruth Nielsen, Connie Damgaard, Karin Henriksen, Annette Brandsholm, and Tina Maagard as well as for the chemical and sensory analysis done by Jens Madsen, Birgitte Foged, Sidsel Jensen and Ulla Kidmose. Furthermore, I would like to thank Monika Schreiner, Susanne Neugart and Antje Bamberg for inviting me to the Department Quality at Leibniz-Institute of Vegetable and Ornamental Crops in Grossbeeren, Germany and helping me do the flavonoid glycoside analyses.

All this work could not have been done without the financial support of The Danish Council for Strategic Research’s Programme Commission on Health, Food and Welfare and Graduate School of Science and Technology, Aarhus University.

Along the journey, my colleagues at the research center in Årslev created a pleasant and inspiring work environment. I enjoyed coming to work every day. Special thanks go to all my PhD colleagues who made everyday life colorful and rewarding due to our different backgrounds and cultures. Further thanks are due to Anders Kjær, Tove Kjær Beck, Maria Obel Thomsen and Sylvia Travers for sharing thoughts, both big and small.

And last of all, I am grateful for the support of my family, family in law and my dearest Mads, Silje and Gry.

Marie Grønbæk
June 2014
Abstract

White cabbage (*Brassica oleracea* L. var. *capitata*) and curly kale (*Brassica oleracea* L. var. *sabellica*) crops are grown worldwide and known for their high content of health beneficial phytochemicals, particularly glucosinolates (GLSs) but also phenolic compounds. These phytochemicals have been linked to i.a. anti-carcinogenic properties as well as antioxidant activity. The same phytochemicals were associated with the distinct bitter and pungent taste characteristic of cabbages. The concentration of GLSs and phenolic compounds were previously found to be affected by nitrogen (N) and sulfur (S) fertilization as well as variety, cultivar selection, and plant developmental stage. Frost is expected to changes the sensory properties of kale. Therefore, the aim of this PhD project was to gain more knowledge on how cultivation strategies in relation to plant agro-biological conditions affect the phytochemical composition, concentration and thereby the sensory properties of cabbage.

One greenhouse and three field trials with split N and S dose fertilization, increasing application of N and S fertilizers, cultivar selection, harvest time throughout the cultivation period, and frost exposure were conducted. Analyses were performed for GLSs, flavonoid glycosides, hydroxycinnamic acid derivatives, polyphenols, soluble sugars, plant N and S concentration, dry matter, growth pattern and yields, as well as sensory profiling.

Results showed that split N dose fertilization increased indole and total GLS concentration in white cabbage, whereas concentration of aliphatic GLSs decreased and kaempferol glycosides increased in curly kale when compared to non-split fertilization. Increased S fertilization enhanced GLS concentration in both varieties, but split S dose fertilization did not affect GLSs. The kale F1 hybrid ‘Reflex’ showed higher responsiveness to increased application of N fertilizers compared to two traditional cultivars ‘Høj Amager Toftø’ (HAT) and ‘Halvhøj Ekstra Moskuset Tiara’ (Tiara). Lower crop yield and higher plant N concentration for Tiara compared to higher crop yield and lower plant N concentration and diverse biomass allocation for ‘Reflex’ and HAT were congruent with higher and lower phytochemical concentrations, respectively. Frost exposure did not alter phytochemical composition and concentration but changed sugar composition. Moreover, ontogeny and cold acclimation increased sugar content and flavonoid glycoside concentration in 13 week old plants and GLS concentration in 17 week old plants. The sensory profiling confirmed the phytochemical stability to frost exposure as the most significant effect of frost was texture alterations. Cultivar selection and N and S fertilization approaches resulted in kales
with different sensory profiles. However, the concentration of phytochemicals could not predict the perception of bitterness, astringent and pungency, but sugar content increased the sweet perception of the kale.

In conclusion this PhD project has contributed new knowledge on fertilizer strategies with respect to altering GLS and flavonoid glycoside composition and concentration as well as the sensory profile of white cabbage and curly kale. Furthermore, cultivar selection proved an important factor when testing these strategies, which implies a need for additional focus and development of fertilizer management adjusted to the selected cultivar. Plant ontogeny should be taken into account depending on the phytochemical of interest, as GLS and flavonoid glycoside concentrations had different optimums throughout the cultivation period. We conclude that cultivar selection and fertilizer strategy, but not frost exposure, produce white cabbage and curly kale with great diversity in terms of phytochemical and sensory properties. However, phytochemical composition and concentration cannot be used to predict the sensory properties. This opens up perspectives for producing health beneficial cabbage and curly kale through plant ontogeny at harvest time, cultivar selection and fertilization strategy.
Resumé (in Danish)

Verden over bliver hvidkål (*Brassica oleracea* L. var. *capitata*) og grønkål (*Brassica oleracea* L. var. *sabellica*) dyrket som afgrøder. De er kendt for at indeholde sundhedsgavnlige indholdsstoffer, specielt glukosinolater (GLS), men også fenolforbindelser. Der er fundet sammenhænge mellem indtag af disse antioxiderende indholdsstoffer og lavere risiko for at udvikle visse kræftsygdomme. Indholdsstofferne giver desuden kål den karakteristiske den bitre og skarpe smag. Øget tilførsel af nitrogen (N) og svovl (S) påvirker koncentrationen af GLS og fenolforbindelser i kål, ligesom valg af kæltyp, sort og plantens udviklingsstadium når den høstes kan have indflydelse på indholdsstofferne. Også frost forventes at ændre på grønkålens sensoriske profil.

Formålet med dette ph.d.-projekt var at skabe ny viden om hvordan dyrkningsstrategier i relation til plantens agro-biologiske betingelser påvirker koncentrationen og sammensætningen af indholdsstoffer foruden kålens sensoriske profil.


Resultaterne viste at delt gødskning med N fik koncentrationen af indol-GLS og totale GLS til at stige i hvidkål, mens forskellige af alifatiske GLS faldt og koncentrationen af kæmpferol glykosider steg i grønkål sammenlignet med udelt gødskning. Øget tilførsel af S fik koncentrationen af GLS til at stige i begge kæltyper, mens delt S gødskning ikke havde en effekt. Grønkålssorten 'Reflex' (F1 hybrid) reagerede kraftigere på øget N-tilførsel sammenlignet med de to gamle sorter 'Høj Amager Toftø' (HAT) og 'Halvhøj Ekstra Moskruset Tiara' (Tiara). Tiara havde et lavere udbytte, et højere N-indhold og en højere koncentration af GLS sammenlignet med 'Reflex' og HAT, som havde et højere udbytte, et lavere indhold af N i planten, forskellige vækstmønstre og en tilsvarende lavere koncentration af GLS. Frosteksponering havde ingen effekt på koncentrationen og sammensætningen af indholdsstoffer, men ændrede forholdet mellem sukker og fruktose.

Desuden var plantens udviklingsstadium og tilpasning til kulde medvirkende til at koncentrationen af sukker og flavonoid glycosider steg i planter indtil planterne var 13 uger gamle, mens koncentration af GLS steg fra udplantning og indtil planterne var 17 uger. Den sensoriske profilering bekræftede den manglende effekt af frosteksponering på

Publications

Paper I.


Paper II.


Paper III.

Influence of cultivar and fertilizer approach on curly kale (*Brassica oleracea* L. var. *sabellica* L.). I. Genetic diversity reflected in agronomic characteristics and phytochemical concentration. Groenbaek, M., Jensen, S., Neugart, S., Schreiner, M., Kidmose, U. and Kristensen, H.L. Accepted for publication in Journal of Agricultural and Food Chemistry, a revised version is in press. Submitted as a joint manuscript with paper IV.

Paper IV.

Influence of cultivar and fertilizer approach on curly kale (*Brassica oleracea* L. var. *sabellica* L.). II. Variations in the content of phytochemicals and how it is reflected in the sensory properties. Jensen, S., Groenbaek, M., Beck, T. K., Kristensen, H.L., Edelenbos, M. and Kidmose, U. Has been rewritten and resubmitted after an invitation to Journal of Agricultural and Food Chemistry. Submitted as a joint manuscript with paper III.
Abbreviations

APS: adenosine 5’-phosphosulfate
BONA: Halvhøj Kruset Bona
C: carbon
CV: cultivar
DAT: days after transplanting
DIT: diacylated tetraglycosides
DM: dry matter
DW: dry weight
FC: Folin Ciocalteu
FW: Fresh Weight
GA: Gallic acid equivalents
GDA: Generic Descriptive Analysis
GLB: glucobrassicin
GLI: glucoiberin
GLN: gluconapin
GLR: glucoraphanin
GLS: glucosinolate
GST: gluconasturtiin
HAST: Høj Amager Sundby Torve
HAT: Høj Amager Toftø
HKK: Halvhøj Kruset Konserva
HY: 4-hydroxy glucobrassicin
K: potassium
LAVO: Lav Opretvoksende
LKH: Lav Kruset Harvester
LS: least squares
MAM: methylthioalkylmalate synthase
MATT: monoacylated tri- and tetraglycosides;
ME: 4-methoxy glucobrassicin
MeOH: methanol
MYB: myeloblastosis
N: nitrogen
NGLB: neoglucobrassicin
P: phosphorus
PAL: phenylalanine ammonia lyase
PAPS: 3’-phosphoadenosine 5’-phosphosulfate
PAR: photosynthetically active radiation
PPFD: photosynthetic photon flux density
PRO: progoitrin
QTL: quantitative trait loci
ROS: reactive oxygen species
S: Sulfur
SIN: sinigrin
TIARA: Halvhøj Ekstra Moskruset Tiara
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Chapter 1

1.1 Thesis Introduction

This PhD project is a part of a multi-institutional project with the title: “Maximizing the taste and health value of plant food products – impact on vegetable consumption, consumer preferences and human health factors” (MaxVeg). The overall objective of the project is to develop new tools and strategies to increase consumption and production of bitter and strong tasting root vegetables and cabbages with high phytochemical content and specific consumer groups targeted for high consumer preferences. A high phytochemical content is thought to influence both health value and the bitter taste of vegetables (Björkman et al. 2011, Drewnowski and Gomez-Carneros 2000, Verhoeven et al. 1997). However, modern cultivars might have been bred with the aim of producing sweeter and less bitter vegetables in order to meet consumer preferences, since taste is a major influence when selecting food (Drewnowski and Gomez-Carneros 2000). This means that newer cultivars potentially possess less health value. The possibility of altering the phytochemical content through cultivar selection and production in order to create a healthy and appealing product with respect to taste was the applied question behind the overall project. In more detail, the main focus of this PhD project was to investigate how cultivation strategies affected the phytochemical content in old traditional and new cabbage (Brassica oleracea L.) cultivars.

Therefore the aim of the present PhD project was to generate new understanding of the agro-biological conditions which affect the phytochemical concentration and, consequently, taste of cabbages. In order to identify possibilities for producing a crop with potential health and sensory value, the focus has been on testing different cultivation strategies in relation to, in particular, glucosinolate (GLS) and flavonoid glycoside concentration. Two experimental setups were chosen: a trial in the greenhouse with potted white cabbage (B. oleracea L. var. capitata) plants, where the effect of split dose fertilization strategy was tested, and three full scale field trials with curly kale (B. oleracea L. var. sabellica). These included further fertilizer experiments with N level and timing in addition to S level, cultivar selection and controlled frost exposure. The curly kale sensory properties were evaluated by a sensory panel. A schematic overview of the experiments, analyses, and discussion points addressed in the different chapters is presented in Figure 1. Although field trials can be challenging in the sense of a less controlled environment and increased variation, this setup was chosen in order to make the results more applicable to
growers and retain a realistic environment when looking for new tools and cultivation methods for future food production.

The following sections of the thesis will give the reader an introduction to the background of the topics addressed in Chapters 2 to 5. A discussion on all of the studies reported is presented in Chapter 6, followed by the conclusions and perspectives in Chapter 7.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Analyses</th>
<th>Discussion points</th>
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<td><strong>Greenhouse</strong></td>
<td>N split dose</td>
<td>Glucosinolate</td>
<td><strong>Paper I</strong></td>
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<tr>
<td>White cabbage</td>
<td>S level</td>
<td>Plant N and S</td>
<td>N &amp; S split effects</td>
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<td></td>
<td>S split dose</td>
<td>Soil N and S</td>
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<td></td>
<td></td>
<td>Biomass yield</td>
<td>NH₄+/NO₃- soil ratio</td>
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<tr>
<td><strong>Field 1</strong></td>
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<td>Curly kale</td>
<td>S level</td>
<td>Plant N and S</td>
<td>N fertilizer strategy effects</td>
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<td></td>
<td>Frost exposure</td>
<td>Flavonoid glycoside</td>
<td>Plant developmental stage</td>
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<td></td>
<td>Harvest time</td>
<td>Sugar</td>
<td>Frost effects on phytochemicals</td>
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<td></td>
<td>Soil N</td>
<td>Frost effects on sensory</td>
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<td><strong>Field 2</strong></td>
<td>N level</td>
<td>Glucosinolate</td>
<td><strong>Paper III and IV</strong></td>
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<tr>
<td>Curly kale</td>
<td>Cultivar</td>
<td>Flavonoid glycoside</td>
<td>Growth pattern</td>
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<td></td>
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<td>Polyphenols</td>
<td>Primary/secondary metabolism</td>
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<td>Sugar</td>
<td>CV effects on phytochemicals</td>
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<td></td>
<td>Plant N and S</td>
<td>CV effects on sensory</td>
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<td></td>
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<td>Biomass allocation</td>
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<td></td>
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<td>Sensory evaluation</td>
<td></td>
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<tr>
<td><strong>Field 3</strong></td>
<td>N level</td>
<td>Glucosinolate</td>
<td><strong>Paper III and IV</strong></td>
</tr>
<tr>
<td>Curly kale</td>
<td>S level</td>
<td>Sugar</td>
<td>N &amp; S interaction</td>
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<td></td>
<td></td>
<td>Plant N and S</td>
<td>S effects on glucosinolates</td>
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<td></td>
<td></td>
<td>Biomass allocation</td>
<td>Fertilization effects on sensory</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sensory evaluation</td>
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</tr>
</tbody>
</table>

Figure 1. Schematic overview of the conducted experiments, treatments, analyses and discussions addressed. N: nitrogen; S: sulfur; CV: cultivar.

The aim of the thesis is to present the scientific work conducted throughout the PhD project with some background to set the scene, giving an introductory literature review of the “state-of-the-art” within the background areas.
1.2 Brassica Vegetables

The broad range of Brassica vegetables belongs to the Brassicaceae family; they are all thought to descend from the wild Brassica oleracea (Snogerup et al. 1990). The group of Brassica oleracea vegetables is very diverse by the fact of their looks and the different plant parts consumed within each variety: immature flowers in broccoli (B. oleracea L. var. italica) and cauliflower (B. olearacea L. var. botrytis), leaves in cabbages and kales and axillary buds in brussels sprouts (B. oleracea L. var. gemmifera). Furthermore, cultivars within each variety have been developed through controlled breeding, resulting in e.g. higher yield, uniform growth and better disease resistance with time. However, the old cultivars have regained attention due to possibly diminished traits such as sensory properties, agronomic qualities or health value in the form of phytochemicals (Cartea et al. 2008, Kopsell et al. 2007, Schmidt et al. 2010a). At the Nordic Genetic Resource Center (NordGen 2014) genetic diversity within plant food and agriculture is conserved as i.a. seeds or living plant material. The traditional cultivars used in the MaxVeg project were obtained from NordGen. For this PhD project, white cabbage and curly kale were chosen as main crops – two leafy Brassicas which are normally harvested during autumn or winter in Denmark and not grown as biannual crops. The Brassica vegetables have been studied intensely within the last couple of decades, especially because of the phytochemicals and their potential health value (Fahey et al. 2001). However, curly kale has been explored less and very few field studies have been conducted. In 2012 the production of cabbages (all B. oleracea and B. rapa varieties) amounted to 134*10^6 tons in global terms, whereas Denmark accounted for 34,884 tons or 0.03% of the world production (FAOSTAT 2014). From these, approx. 19,000 tons were white cabbage, whereas kale amounted to 1,100 tons in 2012 (The Danish AgriFish Agency 2014). In comparison, 451*10^6 tons of potatoes were produced globally. Seen from this perspective, the Brassica vegetables are less dominant, yet still important as a source of healthy phytochemicals, vitamins, and minerals, as well as energy.
1.3 Phytochemicals in Cabbage and Kale

The term phytochemical basically means plant chemical and covers plant-derived compounds which might affect human health but are not essential nutrients like proteins or vitamins. Different phytochemicals are represented in cabbage: carotenoids, chlorophyll, GLSs, polyphenols and fibers among others (Higdon 2007). The main focus of this project has been on GLSs, flavonoid glycosides, and hydroxycinnamic acid derivatives, for which reason their biology will be described in the following sections.

GLSs are phytochemicals which contain S and are not only found within the Brassicaceae. In more than 500 species of dicotyledonous angiosperms, up to 120 different GLSs have been identified (Fahey et al. 2001). When plant tissue is disrupted the GLS molecule is hydrolyzed: myrosinase cleaves off the glucose and the sulfate group is spontaneously lost, resulting in the formation of isothiocyanates, thiocyanates or nitriles. This process causes the pungent smell and taste known in cabbages. These breakdown products act both as repellents against herbivores and as attractants to pollinators (Halkier and Gershenzon
The GLSs are grouped according to their basic structure and the amino acid precursor: aliphatic GLSs are derived from alanine, leucine, isoleucine, methionine or valine; indole GLSs derive from tryptophan; and aromatic GLSs are derived from phenylalanine or tyrosine. The majority of aliphatic GLSs identified in *Brassicaceae* derive from methionine (Kliebenstein et al. 2001).

In short, the GLS biosynthesis can be described in terms of three steps:

1) Side chain elongation of the amino acid, where the parent amino acid is deaminated before it enters a cycle with acetyl-CoA condensation, isomerization and oxidation-carboxylation, respectively, gaining one carbon (C) atom per round.

2) Glucone biosynthesis with addition of glucose via UDP-glucose, resulting in the core GLS skeleton.

3) Modifications of the *R* group, especially in methionine derived GLSs (Halkier and Gershenzon 2006).

Table 1. contains the common names of the GLSs identified within the present project. Chemical names and structures of the *R* group are also presented there.
Table 1. Common names of GLSs and structural formulas of their $R$ groups identified in white cabbage and curly kale in the PhD project.

<table>
<thead>
<tr>
<th>Basic structure of GLS</th>
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<tr>
<td><img src="image" alt="GLS Structure" /></td>
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<table>
<thead>
<tr>
<th>Common name</th>
<th>Chemical name of $R$ group</th>
<th>Structure of $R$ group</th>
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<tbody>
<tr>
<td><strong>Aliphatic GLS</strong></td>
<td></td>
<td></td>
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<tr>
<td>Glucoiberin</td>
<td>3-methylsulphinylpropyl</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Progoitrin</td>
<td>$(R)$-2-hydroxy-3-butenyl</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Glucoraphanin</td>
<td>4-methylsulphinylbutyl</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Sinigrin</td>
<td>2-propenyl</td>
<td><img src="image" alt="Structure" /></td>
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<tr>
<td>Gluconapin</td>
<td>3-butenyl</td>
<td><img src="image" alt="Structure" /></td>
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<tr>
<td><strong>Indole GLS</strong></td>
<td></td>
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<tr>
<td>4-Hydroxy glucobrassicin</td>
<td>4-hydroxy-3-indolylmethyl</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Glucobrassicin</td>
<td>3-indolylmethyl</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>4-Methoxy glucobrassicin</td>
<td>4-methoxy-3-indolylmethyl</td>
<td><img src="image" alt="Structure" /></td>
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<tr>
<td>Neoglucobrassicin</td>
<td>1-methoxy-3-indolylmethyl</td>
<td><img src="image" alt="Structure" /></td>
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<td><strong>Aromatic GLS</strong></td>
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<tr>
<td>Gluconasturtiin</td>
<td>2-phenylethyl</td>
<td><img src="image" alt="Structure" /></td>
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</table>
Flavonoid glycosides are a sub-group of the broad class of plant phenols and have a shielding effect against ultra violet light, as well as an antioxidant capacity (Edreva 2005, Higdon 2007). In the biosynthesis of flavonoids, phenylalanine is the main amino acid substrate (Hahlbrock 1979). Conversion of phenylalanine to the flavonoid precursor flavanone naringenin goes through phenylalanine ammonia lyase, cinnamate-4-hydroxylase, chalcone synthase and chalcone isomerase i.a. (Schmidt et al. 2010a). The basic structure of flavonoids consists of one aromatic ring and a heterocyclic ring system with an oxygen atom (Figure 2A). The main flavonoid aglycones found in kale are kaempferol and quercetin, as well as isorhamnetin to a lesser extent (Schmidt et al. 2010b). The aglycones are characterized on the basis of the side-chain of the B ring (R¹; Figure 2A). Kaempferol is the precursor of quercetin from which isorhamnetin is derived via a methylation (Schmidt et al. 2010a). In plants, flavonoids normally occur as conjugates with glucose. Kaempferol and quercetin have been found glycosylated with up to six glucose moieties in tronchuda cabbage (*B. oleracea* L. var. *costata* DC.) (Ferreres et al. 2005, Sousa et al. 2008). Normally, a part of the flavonoid glycosides found in kale is acylated with different hydroxycinnamic acids (Figure 2B) (Olsen et al. 2009, Schmidt et al. 2010b). The phenylpropanoid moieties of hydroxycinnamic acids have the same origin as flavonoids, and hydroxycinnamic acids can be found independently in kale (Hahlbrock and Grisebach 1979).

![Figure 2](image-url)

**Figure 2.** Flavonoid aglycone skeleton (A) and hydroxycinnamic acids (B) found in curly kale (Schmidt et al. 2010b).

<table>
<thead>
<tr>
<th>Compound</th>
<th>R¹</th>
<th>R²</th>
<th>R³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaempferol</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Quercetin</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Isorhamnetin</td>
<td>OCH₃</td>
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1.3.1. Health Potential of Brassica Vegetables

Several reviews have addressed the health aspects of the Brassica vegetables and they roughly agree on a positive response to human health when consuming Brassica vegetables (Björkman et al. 2011, Jahangir et al. 2009, Latte et al. 2011, Verkerk et al. 2009). For GLSs it is mainly the breakdown product; i.e. nitriles, isothiocyanates and thiocyanates that exhibit bioactivity; however, intact GLSs have also shown effects (Latte et al. 2011). Both epidemiological and in vitro studies indicate preventive effects of GLSs, especially with respect to certain types of cancer, but reduced cardiovascular disease mortality and anti-inflammatory effects have also been documented (Hwang and Lee 2006, Jiang et al. 2013, Marconett et al. 2012, Verkerk et al. 2009, Zhang et al. 2011). Thus, studies on tumor growth in cell systems have shown inhibitory effects of the glucoraphanin breakdown product sulforaphane on prostate, breast and colon cancers (Björkman et al. 2011). The same isothiocyanate has been suggested as reducing carcinogen activation in general, whereas mutagenic effects of the indole neoglucobrassicin have been found, for which reason an attempt should be made to down-regulate it (Latte et al. 2011). Further benefits of aliphatic GLS breakdown products were found from progoitrin nitriles thought to protect against the formation of colon cancer tumor in mice, as well as inhibition of metastasis by the sinigrin isothiocyanate in human cell culture studies (Barrett et al. 1998, Hwang and Lee 2006). Regarding the group of indole GLSs the glucobrassicin indole-3-carbinol revealed inhibition of breast cancer proliferation in human cell cultures as well as in vitro and in vivo anti-inflammatory effects (Jiang et al. 2013, Marconett et al. 2012). The gluconasturtiin breakdown product phenylethyl isothiocyanate, together with sulforaphane, likewise showed down-regulation of markers of inflammation (Björkman et al. 2011). Although negative effects of e.g. goitrin on thyroid metabolism have been documented, the benefits from consuming Brassica vegetables exceed the negative effects, taking into account the relatively low daily intake of indole GLSs assumed to be approx. 5 mg/day in Germany (Björkman et al. 2011, Latte et al. 2011, Steinbrecher and Linseisen 2009).

The phenolic compounds are the most important phytochemicals among the ones having antioxidant capacity (Jahangir et al. 2009). Furthermore, a vast number of health properties of phenolics and flavonoids in Brassica vegetables have been reported. An intervention study found that higher quercetin intake for men resulted in reduced lung cancer incidence as well as asthma incidence for both genders (Knekt et al. 2002). Furthermore, quercetin showed higher antioxidant activity than kaempferol (Kim et al.
2006, Zietz et al. 2010), possibly due to the dihydroxylated B-ring, which might contribute
to prevention of e.g. cancer, atherosclerosis and chronic inflammation by slowing down the
oxidative degradation (Cartea et al. 2011). The type of hydroxycinnamic acid acylating the
flavonoid glycoside might also influence antioxidant activity. Thus, the dihydroxy structure
of caffeic acid conjugated to kaempferol glycosides in Aconitum sp. was suggested as
increasing radical scavenging activity (Braca et al. 2003). However, these findings were not
supported by the study of Zietz et al. (2010), as they found no effects of the conjugated
hydroxycinnamic acid, but a higher antioxidant activity of the quercetin glycosides
compared to kaempferol in curly kale. Quercetin glycosides moreover revealed a better
absorbance in the human small intestine compared to quercetin aglycone (Hollman et al.
1995). Quercetin, kaempferol, and isorhamnetin aglycones exhibited anti-inflammatory
effects (Hämäläinen et al. 2007) and synergistically effects of quercetin and kaempferol
flavonols were found to repress cell proliferation in human gut cancer cells, underlining
the possibility that single phytochemicals may have certain effects in specific assays but
different properties when consumed in combinations, like their occurrence in fruits and
vegetables (Ackland et al. 2005).

1.4 Cultivation Strategies as a way to Modulate Phytochemical
Concentration in Cabbage and Kale

Strategies on altering GLS and flavonoid concentrations in Brassica crops have been
studied widely. The major areas have been the effects of harvest time/season (Aires et al.
2013, Steindal et al. 2013), irrigation (Radovich et al. 2005, Zhang et al. 2008), fertilizer
form (Fallovo et al. 2011, Kim et al. 2006) and timing (Paper I, Paper II), light level or
source (Fallovo et al. 2011, Neugart et al. 2013), cultivar selection (Charron et al. 2005,
Paper III, Paper IV, Schmidt et al. 2010a, Vallejo et al. 2003b), location of growth (Velasco
et al. 2007), and plant developmental stage (Paper II, Pereira et al. 2002, Vallejo et al.
2003a). In this study, the focus has been on fertilizer levels and timing, cultivar selection,
plant ontogeny at harvest time, and temperature influence in the sense of frost exposure.
In the following paragraphs I will go through the effect of these parameters in relation to
the phytochemicals described in the previous section.
1.4 Cultivation Strategies as a way to Modulate Phytochemical Concentration in Cabbage and Kale

1.4.1. Nitrogen and Sulfur Fertilizer Effects on Growth and Phytochemicals

All plants need a certain level of N to sustain primary growth. Cabbage and kale in general have a high demand for N and growth studies documented continuous increase in biomass of white cabbage up to a level of 960 kg N ha⁻¹ (Sorensen 1999). Furthermore, cabbages have the ability to take up more N than needed for primary growth: a luxury uptake (Maynard et al. 1976). Application of S fertilizers to Brassica vegetables is standard practice due to a relatively high demand for S compared to other crops, e.g. cereals (Schnug and Evans 1992). This is mainly due to the GLS biosynthesis, but also biomass yield of Brassica vegetables are affected by S level (Paper I, Rosen et al. 2005, Schonhof et al. 2007). N fertilizer strategies are highly relevant with respect to higher yield/optimal plant growth, nutrient use efficiency, and for environmental purposes (Kitchen and Goulding 2001). Therefore splitting the N application into more than one dose is standard agricultural practice in order to optimize fertilizer availability throughout plant growth period. During this PhD project the effect of split dose N and S fertilization was investigated. The results are reported in Paper I and II.

![Two month old kale plants grown under one N dose (left hand) and split N dose (right hand) conditions, with the second dose to be applied the day the picture was taken. Photo: Marie Grønbæk.](image)

As phytochemicals are characterized as secondary metabolites, non-essential for main plant growth, the balance between primary and secondary metabolism is of great importance, particularly in the study of fertilizers essential to primary growth, as N and S are. In relation to this balance, the carbon-nutrient hypothesis (Bryant et al. 1983) suggests that in plants experiencing N deficiency but normal CO₂ levels, the C-based phytochemical concentration increases due to excess C. Conversely, N-sufficient plants with reduced CO₂ availability increase in N-based phytochemical concentration as a result of excess N. However, it has been suggested that this hypothesis is over-simplified and it has been
argued that C and N metabolism changes are parallel and not antagonistic (Fritz et al. 2006). Molecular studies on gene expression in N-deprived arabidopsis (*Arabidopsis thaliana*) and tobacco plants have revealed a significant activation/up-regulation of several genes assigned to photosynthesis, chlorophyll synthesis, and plastid protein synthesis, as well as genes regulating secondary metabolism (Fritz et al. 2006, Lea et al. 2007, Scheible et al. 2004). They further concluded that N deficiency increased the metabolism of C-rich phytochemicals and decreased the N-based phytochemical nicotine, supporting the carbon-nutrient hypothesis (Fritz et al. 2006, Lea et al. 2007). On the other hand, studies on different *Brassica* species grown under elevated CO₂ levels and different N application revealed species-specific responses both supportive of and contradictory to the carbon-nutrient hypothesis and stated that the C/N ratio of the plants investigated could not be used as a predictive tool for plant phytochemical status (Karowe et al. 1997, La et al. 2009).

The N and S link primary and secondary metabolism through amino acids, as the amino acids are both constituents in proteins and act as precursors for the GLS and flavonoid biosynthesis. Additionally, several enzymes play major roles in the assimilation and reduction of the inorganic sulfate and nitrate absorbed by the plants (Figure 3) (Brunold 1993, Mugford et al. 2011). These processes are closely connected, as adenosine 5´-phosphosulfate (APS) reductase is controlled by N availability on a transcriptional level (Koprivova et al. 2000), and nitrate reductase activity was found to decrease when S availability was low (Brunold 1993).
1.4 Cultivation Strategies as a way to Modulate Phytochemical Concentration in Cabbage and Kale

Figure 3. Scheme of assimilatory nitrate and sulfate reduction in plants. APS: adenosine 5´-phosphosulfate; PAPS: 3´-phosphoadenosine 5´-phosphosulfate (modified from Brunold et al. 1993 and Mugford et al. 2011).

1.4.2 Glucosinolate Modulation by Nitrogen and Sulfur Fertilizers

The partitioning of S in GLS molecules and amino acid precursor, methionine, makes this fertilizer an obvious candidate of interest. Besides the close connection between nitrate and sulfate assimilation, plant sulfate metabolism balances between incorporation into cysteine leading to methionine in the primary assimilation. And secondary assimilation resulting in active sulfate (3´-phosphoadenosine 5´-phosphosulfate, PAPS) used for sulfation in the last step of GLS synthesis (Figure 3) (Mugford et al. 2011). A vast number of studies confirm the importance of S fertilization in relation to GLS biosynthesis. The majority found increasing levels of GLSs when cultivated under increased S fertilization (Omirou et al. 2009, Paper I, Paper III, Rosen et al. 2005, Schonhof et al. 2007, Stavridou et al. 2012).

Given the closely connected assimilation of nitrate and sulfate, in addition to their common contributions to both primary and secondary metabolism, an interactive effect of the fertilizers on GLS biosynthesis and concentration seems plausible. These interactive
effects have been documented in different *Brassica* species and plant tissues (Gerendas et al. 2008, Paper I, Schonhof et al. 2007). The investigations were performed in potted plants under greenhouse conditions, whereas field trials with corresponding treatments of N and S supply showed fewer interactive effects (Paper III, Rosen et al. 2005, Stavridou 2012). In general, differing results with respect to N level and timing effects on GLS biosynthesis have been found; decreased, increased, and unchanged individual and total GLS concentrations under enhanced and split dose application of N fertilizers (Jones et al. 2007, Omirou et al. 2009, Paper I, Paper III, Stavridou et al. 2012). The contradicting results point to a complex regulation of the GLS biosynthesis, as N plays essential roles in many vital plant processes. This complexity was supported by molecular studies in arabidopsis, which showed no clear relationship between GLS biosynthesis genes transcript level and the GLS accumulation in the plant (Sønderby et al. 2010). The form of N was also proven to have an impact on GLS concentrations within *Brassicaceae*; increased NH$_4^+$/NO$_3^-$ ratio increased the aliphatic, indole, and total GLS concentrations in *B. rapa*, *B. juncea*, and *Eruca sativa* Mill. (Fallovo et al. 2011, Kim et al. 2006). Furthermore, NH$_4^+$ provided for arabidopsis increased the leaf cysteine concentrations containing S, when compared to NO$_3^-$ supply (Koprivova et al. 2000), which takes us back to and confirms the close connection between N and S fertilizers once more.

### 1.4.3 Flavonoid and Hydroxycinnamic Acid Modulation by Nitrogen Fertilizers

The phenylalanine ammonium lyase (PAL) enzyme constitutes one of three essential steps in the phenylpropanoid metabolism leading to flavonoid biosynthesis (Hahlbrock and Grisebach 1979). As a branching point of the polyphenol biosynthetic pathway, the enzyme has been widely studied, although only few studies on effects of N fertilization on flavonoid concentration in *Brassica* vegetables have been reported to the best of my knowledge. In general, decreased N levels increased quercetin and kaempferol flavonoid concentrations in different *Brassica oleracea* varieties (Jones et al. 2007, Sousa et al. 2008). Furthermore, Ibrahim et al. (2011) found that enhanced N application resulted in reduced PAL activity, lower total flavonoid concentration and a positive relationship between the two (PAL activity and total flavonoid concentration). Based on molecular studies in N-deficient arabidopsis Lea et al. (2007) revealed increased expression of *PAP1*, *PAP2*, and *GL3* transcription factors, which regulate flavonoid synthesis. Moreover, a clear coordinated response to NO$_3^-$ addition in arabidopsis repressing shikimate pathway was
1.4 Cultivation Strategies as a way to Modulate Phytochemical Concentration in Cabbage and Kale

found. This pathway synthesizes phenylalanine, the phenylpropanoid precursor, and contributes to the C skeleton of flavonoids (Scheible et al. 2004). In tomato plants (*Lycopersicon esculentum* L.), N deficiency was found to decrease chlorophyll content and photosynthetic activity (Guidi et al. 1998). Since flavonoids act as shielding agents possibly by quenching the harmful reactive oxygen species (ROS) i.a., an up-regulation in flavonoid concentration with reduced N availability could be explained by this link (Klimov et al. 2008). Regarding the N fertilizer form, addition of 100% NH$_4^+$ to *B. rapa* and *B. juncea* resulted in decreased kaempferol and quercetin flavonoid concentrations as compared to 100% NO$_3^-$ application possibly caused by enhanced N concentration in the leaves under 100% NH$_4^+$ application (Fallovo et al. 2011). The group of hydroxycinnamic acids in *Brassica* vegetables constitutes a substantial part of the phenolic compounds (Neugart et al. 2012b, Olsen et al. 2010, Sousa et al. 2008, Velasco et al. 2011). However, even fewer studies on the effect of N fertilization on hydroxycinnamic acids have been carried out. Thus, Sousa et al. (2008) found diverse effects of increased N fertilization in 10 hydroxycinnamic acid derivatives in tronchuda cabbage. As hydroxycinnamic acids and flavonoids share many steps in their biosynthetic pathways we might expect similar responses to N fertilizers to some extent. The effect of S fertilization on flavonoids will not be addressed here, as it was not investigated in this study.

1.4.4 Cultivar Effects on Phytochemical Concentration

Genetic variation gives a range of differences which can be exploited for many objectives. These differences were subjected to breeding long before knowledge of the existence of genes, based on crop phenotypes. With a view to improving crops, there has always been major interest in yield and disease resistance throughout all historical periods. The focus on qualities related to phytochemicals and sensory properties i.a. in *Brassica* has increased lately, and traditional landraces as well as hybrid cultivars have been tested regarding these aspects (Brown et al. 2003, Cartea et al. 2008, Charron et al. 2005, Paper III, Paper IV, Rangkadilok et al. 2004, Schmidt et al. 2010a, Vallejo et al. 2003c).

GLS differences both with respect to quantity and quality have been documented with up to 27-fold changes in glucoraphanin and indole GLS concentrations among broccoli and pak choi (*B. rapa* ssp. *chinensis*) cultivars, respectively (Kushad et al. 1999, Wiesner et al. 2013). Furthermore, it has been suggested that indole GLSs are more susceptible to environmental factors, and aliphatic GLS variability among cultivars could
Correspondingly, be explained by genetic variation (Brown et al. 2003, Kushad et al. 1999, Velasco et al. 2007). Wiesner et al. (2013) revealed three groupings of 13 pak choi cultivars based on GLS composition and concentration, partially supported by the relative expression of genes involved in aliphatic GLS biosynthesis. Morphological differences between two of the groups were also found. This resembles the findings by Cartea et al. (2008), where groupings of kale cultivars (acephala group) based on agronomic traits showed different GLS concentrations, and points to the perspective of this PhD project where cultivar genetic differences were reflected in growth pattern and phytochemical concentration (Paper III).

Flavonoid and hydroxycinnamic acid derivatives have also been reported as variable among cultivars; however, fewer studies have been conducted within this area. A comparison among commercial and landrace broccoli cultivars revealed higher levels of total flavonoids and hydroxycinnamic acid derivatives in commercial cultivars (Vallejo et al. 2003b). The opposite trend was documented by Schmidt et al. (2010a) in curly kale, where old traditional cultivars revealed higher flavonoid concentrations compared to modern hybrids. To the best of my knowledge, no studies on flavonoid content reflected in genotypic variation have been conducted with Brassica vegetables. However, a recent investigation of F2 pepper fruits (Capsicum sp.) revealed a clear separation into three clusters based on their metabolite profile, including flavonoids (Wahyuni et al. 2014). For further experiments within cultivar selection for phytochemical qualities and health related aspects, identification of the metabolite quantitative trait locus QTLs is necessary (Hennig et al. 2014).
1.4.5 Temperature and Frost Effects on Phytochemical Concentration

Most *Brassica* varieties cannot survive sub-zero degrees lower than approx. -4 °C, which is probably why most studies with temperature effects on phytochemicals in *Brassica oleracea* have been conducted from around 10 °C upwards (Björkman et al. 2011, Deveci and Aksu 2010). It is a widespread belief among the Danish population that frost before harvest alters kale taste to the better. This was also claimed by Hagen et al. (2009). The idea of altered sensory properties seems plausible in the light of the physiological processes known to occur during cold acclimation in plants. They will be described in what follows.

Soluble sugars accumulate in frost tolerant plants when they are exposed to low temperatures above 0 °C. This process is known as cold acclimation or cold hardening. Soluble sugars are thought to be osmoregulators, keeping the water inside the cell and thereby lowering the risk of ice formation in the apoplast (Theocharis et al. 2012). The accumulation of sugars could possibly increase the sweetness of kale leaves. However, kale possesses other mechanisms to cold acclimate, as antifreeze proteins in cold acclimated kale lowered the level of freezing injury (Atici and Nalbantoglu 1999). Yet, compared to other cold acclimated frost tolerant species, the activity of these proteins was low in kale (Antikainen and Griffith 1997). This means that cold acclimation occurs at lower temperatures above 0 °C, which might result in little or no changes to temperatures actually below 0 °C.

A study on rutabaga roots (*B. napus* ssp. *rapifera*) revealed no changes in total GLS concentration when stored at 0 °C compared to plants kept under initial growth conditions at around 25 °C. But quantitative changes in the GLSs were found (Shattuck et al. 1991). Occasionally frost cell damage might lead to some degree of myrosinase activity and consequently a lower concentration of GLSs – and maybe less bitter taste.

The total flavonol concentration in curly kale dropped 35% after exposure to frost in the field, alongside an increase in sugar content under the same conditions (Hagen et al. 2009). In a study on frost tolerant *Rhododendron*, a positive correlation between high levels of flavonoid glycosides and increased frost resistance has been suggested (Swiderski et al. 2004). Likewise, a clear increase of flavonoids in cold acclimated arabidopsis was found as compared to non-acclimated (Korn et al. 2008). The glycoside of flavonoid or GLSs might delay water crystallization, as described for the soluble sugars, or the scavenging of free radicals by the flavonoid might have an influence. Furthermore, the
1.4 Cultivation Strategies as a way to Modulate Phytochemical Concentration in Cabbage and Kale

relatively high number of hydroxyl groups in the flavonoid molecule should be able to form bonds with water, leaving less possibility of ice formation (Swiderski et al. 2004).

Experimental field covered in snow, February 2012. Photo: Marie Grønbæk

1.4.6 Effects of Plant Developmental Stage on Phytochemical Concentration

Effects of plant developmental stage on GLS concentration have been found in studies of cabbages and kale (Rosa et al. 1996, Vallejo et al. 2003a, Velasco et al. 2007). Increases in indole and aliphatic GLS concentrations were seen in leaves of cabbages and kale before flower initiation, whereas GLS formation in broccoli florets reached an optimum at early stages in contrast to over-maturity.

Investigations of curly kale revealed no differences in total concentration of flavonoids between plants aged from three to six months (Schmidt et al. 2010a). However, increases in quercetin and isorhamnetin and a decrease in kaempferol concentrations were revealed during the cultivation period. Conversely, a higher concentration of total phenolic compounds was shown in five month old plants compared to seven months (Sousa et al. 2008). Both studies addressed the changes to environmental effects, such as UV light and temperature effects, rather than plant age.

In general, ontogenetic effects on phytochemicals might be seen in relation to environmental influences, as they most often interact. Besides the parameters already mentioned, a relationship between GLS concentration and insect pests has been found (Velasco et al. 2007). Furthermore, catabolism of GLSs throughout plant development has also been suggested (Rangkadilok et al. 2002) to mention a few. Low temperatures have been shown to increase quercetin glycosides in kale, possibly due to increasing ROS. Flavonoids are assumed to quench these due to their antioxidant activity (Klimov et al. 2008, Neugart et al. 2012a). In general, the plant benefits from the phytochemical
properties in relation to environmental influences under different developmental stages. Therefore, fluctuations throughout plant development might be seen as the plant’s requirements for e.g. protection or pollinator attraction differences.

1.5 Sensory Properties of Cabbage and Kale

The distinct bitter and pungent taste of cabbage and kale is known to most people. Taste is a main factor in determining food choice, more important than e.g. awareness of health properties (Drewnowski and Gomez-Carneros 2000). The taste profile of cabbage is thus of significant importance in terms of maximizing both health and taste value. Within the field of sensory science, many methodologies have been developed to describe and characterize the sensory differences between products (Lawless and Heymann 1998). Generic Descriptive Analysis (GDA) was used in this project (Paper II and IV) and is an approach which is used to describe a given product by visual appearance, odor, texture and/or flavor attributes in combination (Murray et al. 2001). During GDA and prior to the actual tests, samples which resemble characteristic descriptors of the product to be evaluated are given to a trained panel in order for them to define attributes. A common language and understanding of the attributes chosen relevant to describe the product are then developed by the panel. The actual test takes place in separated booths where each panelist evaluates the samples at their own speed. Here they rank the sample according to the attributes on a linear scale with end point anchors classified as high and low to the right and left, respectively. The panelists have been selected on the basis of having adequate sensory skills and use their senses objectively in the evaluation.

Bitterness of *Brassica* vegetables has mainly been associated with GLSs and their breakdown products. In particular, sinigrin, gluconapin, progoitrin, glucobrassicin, and neoglucobrassicin within the commonly identified GLSs of *Brassica oleracea*, have been mentioned (Pasini et al. 2011, Schonhof et al. 2004, van Doorn et al. 1998). In a study on broccoli and cauliflower cultivars, multiple correlations between the abovementioned GLSs and bitterness were revealed (Schonhof et al. 2004). Likewise, van Doorn et al. (1998) reported a positive correlation between the content of progoitrin and sinigrin and the perceived bitterness of brussels sprouts. In contrast, several studies found no direct relationship between the bitter GLSs and the perceived bitterness of broccoli and turnip greens (*B. rapa* L.) (Hansen et al. 1997, Padilla et al. 2007). However, high total GLS concentration did relate to perception of bitterness in the two studies, indicating that other
GLSs or even phytochemicals are involved in the characteristic taste of *Brassica*. This suggestion was further supported by findings of Zabaras et al. (2013) where total and individual GLS content alone could not explain perceived bitterness of raw broccoli extracts. It is worth mentioning that the bitter taste is the most complex of the five basic tastes, which reflects the ambiguity of the results (Drewnowski 2001).

To date, no studies have evaluated the relationship between flavonoid concentration and bitterness of *Brassica* vegetables, but the general assumption is that phenolic compounds contribute to the bitter taste of plant based food (Drewnowski and Gomez-Carneros 2000). In rocket salad (*Eruca* sp.) perception of bitterness and pungency were positively related to one flavonoid glycoside: kaempferol-3-(2-sinapoyl-glucoside)-4´-glucoside (Pasini et al. 2011).

The content of sugars is also relevant to the overall perception, including the perceived bitterness of *Brassica*, as it has been suggested that perception of bitter and sweet interact. In one study, a significant multiple correlation was found between sweetness perception, the content of sugars and the total concentration of bitter GLSs in broccoli and cauliflower (Schonhof et al. 2004). However, the content of sugars did not influence the perception of sweetness alone and the perception of sweetness appeared to decrease with increasing content of the bitter GLSs. Furthermore, fructose, glucose and sucrose were found to mask the bitter taste of GLSs (Beck et al. 2014, Zabaras et al. 2013), which underlines the complexity of the perception of the bitter taste.

**1.5.1 Cultivation Strategies as a way to Modulate Sensory Properties**

Investigations of N effects on the sensory profile of vegetables are scarce. At present, studies have mostly accounted for effects of N fertilizer form in relation to organic versus conventional cultivation methods (Heeb et al. 2005, Talavera-Bianchi et al. 2010). However, a study on pak choi grown under organic and conventional conditions did study the effects of high, low or no N application on the sensory profile (Talavera-Bianchi et al. 2010). Only small differences were found with respect to the sensory profile; however, an assumed higher overall intensity of sweetness was suggested, but not confirmed, when no N was applied. As GLS concentration is known to increase when S fertilization is applied to *Brassica* vegetables, testing a possible link between S fertilization and the sensory properties of *Brassica* is highly relevant. To my knowledge, this was reported in Paper IV.
for the first time. Studies on onion (*Allium cepa*) revealed an increase in compounds related to pungency when S availability was enhanced (Abbey et al. 2004, Randle 1997).

Cultivar selection has been shown to affect the sensory profiles of different *Brassica* vegetables. Schonhof et al. (2004) documented differences with respect to the attributes bud color, bitter taste and leek like flavor among five broccoli cultivars, whereas broccoli like and cauliflower like flavor differed among the four cauliflower cultivars tested by a professional panel. Furthermore, attributes differed between the two varieties. Similarly, Pasini et al. (2011) examined 37 accessions of rocket salads (*Eruca* sp. and *Diplotaxis* sp.). Here, the trained panel found perceptible differences with respect to bitterness, pungency and aroma intensity i.a. Less pronounced were the findings of a study on eight brussels sprout cultivars, where an expert panel found differences with respect to bitter taste in one out of three trials done in three different years (van Doorn et al. 1998).

With respect to investigations of effects of frost in relation to sensory evaluation, a few references refer to injuries caused by frost and the consequences thereof (olive (Morello et al. 2003) and carrots (Suojala 2000)), or frost storage of processed vegetables (broccoli (Gebczynski and Lisiewska 2006), and brussels sprouts (Olivera et al. 2008)). Seen in the light of cultivation strategies, this is not surprising, as very few vegetable crops, if any, are normally kept in the fields under freezing conditions. The advantage of frost tolerant crops, such as kale, is that growers are not obliged to harvest before the first night frost in October or November, as curly kale is a big part of local traditional Christmas and New Year dishes. Furthermore, it extends the season for fresh vegetables, which also guaranteed a source of i.a. vitamins and food before freezers were invented.

**1.6 Project Hypotheses**

As mentioned, the objective of this PhD project was to generate new understanding of the agro-biological conditions which affect the phytochemical concentration and consequently sensory properties of cabbages. This has led to the following overall hypotheses:

A. The splitting of N fertilizer application to white cabbage and curly kale results in optimized N availability throughout the cultivation period which increases GLS but lowers flavonoid glycoside concentrations as compared to one dose or reduced N fertilization.
B. Sulfur interacts with N in relation to GLS concentration when N is applied in split doses or increasing levels to white cabbage and curly kale.

C. Cultivar selection of curly kale is reflected in growth pattern and thereby phytochemical concentration due to diverse genetic background.

D. Nitrogen and S levels, split N dose, cultivar selection and frost exposure affect the phytochemical concentration and composition and thereby the sensory properties of curly kale.

This is the basis on which the experiments, which are reported in the following 4 chapters, were conducted.

**Chapter 2** reports the influence of N and S split dose fertilization on GLS concentration in white cabbage plants grown in pots under greenhouse conditions. Results showed increased concentrations of indole and total GLSs, but dependent on S fertilization when compared to one dose fertilization.

**Chapter 3** investigates whether split N dose fertilization and frost exposure to curly kale grown in the field alter the composition and concentration of GLSs, flavonoid glycosides, and sugars. Furthermore, the development of phytochemicals throughout cultivation period was assessed. Findings revealed few effects of split dose fertilization and different phytochemical optimums throughout the cultivation period. Frost exposure altered sugar composition but not phytochemical concentration or the related sensory properties.

**Chapter 4** examines the effects of N and S supply and biomass allocation on GLS, flavonoid glycoside, and hydroxycinnamic acid derivatives concentrations in two traditional and one F1 hybrid cultivars of field-grown curly kale. Different responses in growth patterns due to genetic diversity resulted in diverse phytochemical responses among cultivars.

**Chapter 5** evaluates whether a correlation between phytochemical composition and concentration and sensory properties of curly kale can be found. The chapter investigates the possibilities of creating plants with diverse phytochemical composition and concentrations through cultivation methods in the field. The conclusion was that both cultivar selection and fertilizer approaches created diverse samples with respect to phytochemical concentration and sensory properties. The sugar content correlated with the perception of sweetness, but phytochemical concentration did not predict perceived bitterness or astringency.
Chapter 6 will include discussions across the studies of Chapters 2 to 5 with the main hypotheses as starting point. In Chapter 7 the conclusions and perspectives will be presented.
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Chapter 2

2.1 Paper I

Split dose fertilization with urea increases glucosinolate contents in white cabbage (*Brassica oleracea* L. var. *capitata*) under experimental pot conditions

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Published in Scientia Horticulturae, 2014 (168) pp. 64-72.
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A R T I C L E   I N F O

Article history:
Received 20 September 2013
Received in revised form 8 January 2014
Accepted 11 January 2014

Keywords:
Glucosinolates
Split dose
Fertilizer
Nitrogen
Sulfur
Brassica oleracea.

A B S T R A C T

Split dose nitrogen (N) and sulfur (S) applications are a common agricultural practice as safe choices for environmental purposes. However, the effects of split dose practice on glucosinolate (GLS) biosynthesis remain elusive. The objectives of the present study were to investigate the timing effects of N and S fertilizer use on GLS biosynthesis in white cabbage plants. Therefore, timed split and non-split N treatments were combined with three S treatment levels or timed split and non-split S treatments.

Split N dose treatments increased indole and total GLS concentrations, whereas non-split N treatments increased aliphatic GLSs. The effect of S was dependent on N treatment. Split N treatment resulted in enhanced GLS concentrations, which increased from 4351 to 7208 μg g⁻¹ dry weight (DW) with increasing S treatment; and with split and non-split S treatments, GLS concentrations ranged from 5836 to 7208 μg g⁻¹ DW. Non-split N treatment had no effect and GLS concentration was measured at 5510 μg g⁻¹ DW.

Results indicated that equal N availability (split dose) facilitated an increased plant response to S and a subsequent effect on GLS biosynthesis compared to unequal N availability (non-split dose). In terms of practical crop management, the timing of fertilizer addition to white cabbage can be used to optimize GLS concentrations.

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1. Introduction

Nitrogen is an essential plant nutrient that plays a key role in plant growth, and crop yield. Crop production practices within the Brassicaceae plant family routinely apply S as a standard to optimize cabbage growth for human consumption. Both nutrients serve an important role in the biosynthesis of GLSs, which are the characteristic secondary metabolites found in cabbages and members of the Brassicaceae (Verkerk et al., 2009). The enzymatic breakdown products of GLSs are partly responsible for the distinctive taste of cabbage, and also act as a repellent against some herbivores, and attract pollinators (Halkier and Gershenzon, 2006). The GLS structure and chemistry have been thoroughly studied for more than 50 years (Fahey et al., 2001). The GLS investigations have generated great interest due to the health promoting effects of GLS breakdown products. As reviewed by Björkman et al. (2011) and Jahangir et al. (2009), the degradation products were found to reduce developing cancer risks, including colon, bladder, and possibly breast and prostate cancers. Glucosinolate health promoting effects present in Brassica species might be influenced by S supply, and the type and form of N fertilizer available during plant growth, because supply has an impact on GLS content and composition (Björkman et al., 2011; Fallovo et al., 2011). These health aspects provide a motive for further investigations of the GLS biology. Glucosinolates consist of a ‘thioglucose unit’, a ‘sulfonated oxime unit’, and a variable side chain (Gerendas et al., 2009). The variable side chain is often derived from amino acids, and the GLS group is defined by a specific amino acid. Alanine, methionine, valine, leucine, and isoleucine are aliphatic GLS group precursors; phenylalanine and tyrosine are aromatic GLS group precursors; and tryptophan is a precursor to the indole GLS group (Fahey et al., 2001). Glucosinolates are believed to be localized in the cell vacuole, and accumulated in all vegetative and reproductive plant structures (Brown et al., 2003). In addition, evidence indicates GLSs can be transported through plant phloem (Chen et al., 2001). Glucosinolates can be catabolized by the plant...
under low S supply to support primary metabolism, e.g., protein synthesis (Falk et al., 2007). Moreover, studies demonstrated that plant N and S uptake was heavily dependent on a sufficient availability of both soil nutrients (Gerendas et al., 2009; Schonhof et al., 2007). In addition, the amount and type of N and S fertilizer available during growth had an effect on GLS concentration in different Brassica species, however consistent results were not generated (Fallovo et al., 2011; Omriou et al., 2009). The different N forms are interesting when viewed in light of organic cropping systems, because the fertilizers used are often comprised of different types of N-containing compounds.

Optimizing fertilizer availability throughout a plants’ vegetative growth stages is therefore highly relevant to GLS biosynthesis. Optimizing fertilizer availability is standard agricultural practice, and is preferred over a one-dose application. Fertilizer optimization is designed to increase yield and nutrient use efficiency (Kitchen and Goulding, 2001). Optimization in relationship to GLS biosynthesis may likewise be achieved via N and S fertilizers using split-dose treatments, which can ensure a more continuous availability of nutrients over time. However, how this practice affects GLS biosynthesis, or if the timing of fertilizer treatment affects GLS concentrations and composition remains unclear. Therefore, the objectives of this study were to investigate the combined effects of N and S on GLS biosynthesis following one (non-split) or two doses (split) fertilizer treatments during the vegetative growth period in white cabbage, which to our knowledge has not yet been reported in Brassica oleracea.

2. Materials and methods

2.1. Pot experiments

Early white cabbage seeds (B. oleracea L. var. capitata cv. ‘Parel’) were sown on August 10, 2010 in polystyrene boxes filled with a peat growing medium with a low fertilizer level. The boxes were placed in an unheated greenhouse to germinate. Pots (7.5 L) with saucers were filled with a sandy loam (Typic Acrudalf) field soil sieved through a 5 mm mesh, and mixed with 30% sand (8 kg pot−1, total mass, wet weight). The field soil was taken from the top soil layer (25 cm) at Aarslev, Denmark (55°18’N, 10°27’E) containing 1.35% organic C, 33% coarse sand, 38.8% fine sand, 12.6% silt, and 13.4% clay. The soil/sand mixture contained the following DW chemical composition: pH 7.4 (CaCl2, 0.01 M); 26.2 mg inorganic N kg−1; 2.4 mg inorganic S kg−1; 23 mg P kg−1 and 100 mg K kg−1 (P extracted with 0.5 M NaHCO3, K extracted with CH3COONH4). Cabbage plants at the 3–4 leaf stage were transplanted to pots, 24 days after sowing, and watered with tap water (2.8 mg inorganic N L−1 and 35 mg S L−1) as needed throughout the rest of the experimental period. The plants were grown in an unheated greenhouse, with 50 cm distance among pots, and arranged in a randomized complete block design with four replicates per treatment (n = 4). The block design was chosen due to possible spatial variation in the greenhouse conditions. Extra plants (n = 4 per treatment) for the harvest halfway through the experiment were grown under the same conditions. During the growing period, the mean temperature was 15.7 °C with 6.6 and 28.5 °C as minimum and maximum temperatures. The plants were provided with artificial light (PPFD 47 μmol m−2 s−1) from dawn to dusk, when the outdoor light level did not exceed PPFD 117 μmol m−2 s−1. Six days after transplanting (DAT), 1.8 g of N was applied to each pot using granulate urea (CO(NH2)2), 46% N, Yara, Denmark) to ensure sufficient nutrients for proper initial plant growth. Granulate kieserite (MgSO4, 20% S, K+S Kali GmbH, Germany) was used as an S source. Both nutrients were applied manually to the soil, and watered shortly afterwards. All plants were sprayed with an insecticide (Pirimor) at 12 DAT due to a few cases of aphid attack. At 27 DAT (7–8 leaf stage), the different timing and fertilizer level treatments were initiated. The plants were administered 7.8 g N pot−1 in total in the form of urea during the entire experimental period but in different doses and at different time intervals. The following two experiments were conducted simultaneously: Experiment I) split-dose treatment with N (two times 3 g pot−1, applied 27 and 40 DAT, respectively) vs. non-split dose treatment with N (one times 6 g pot−1, applied 40 DAT) combined with three different S levels (0, 0.75 and 1.5 g pot−1, applied 40 DAT); and Experiment II) split-dose treatment with S (two times 0.75 g pot−1, applied 27 and 40 DAT, respectively) vs. non-split dose treatment with S (one times 1.5 g pot−1, applied 40 DAT). The N combinations in Experiment II were as in Experiment I. Because the highest S level in Experiment I was the same treatment as Snon-split in Experiment II, this provided an overlap in N treatments between the two experiments. Halfway through the experiment, 40 DAT (12–14 leaf stage), one plant per replicate (n = 4 per treatment) was harvested for analysis. Soil from two replicate pots representing each treatment 40 DAT was collected after mixing the entire pot soil content. The sample was frozen at −18 °C until analysis. The 2nd treatment was subsequently initiated 40 DAT. At 54 DAT one plant per replicate (n = 4 per treatment) was harvested, and one soil sample from each pot (n = 4 per treatment), was obtained the following day, using the same method as described above. The total aboveground biomass from each pot was weighed immediately after harvesting, chopped, mixed, and a representative sample of approximately 50 g was immediately frozen in liquid nitrogen, and placed in a freezer (−24 °C) before freeze-drying. After freeze-drying, the samples were ground in a mill, and used for GLS analyses. Immediately after harvest, the remainder of the plant material was oven dried at 80 °C to a constant weight, and DW was calculated. The material was used for N and S analyses.

2.2. Plant and soil analyses

The VDLUFA methods were used to determine plant N and S, and soil NO3−, NH4+, and inorganic S (VDLUFA, 1976a, 1976b, 1991).

2.3. Glucosinolate analyses

The GLS analyses followed Krumbein et al. (2005), with some modifications. Plant material was extracted with 9 ml 70% methanol (Sigma-Aldrich, Steinheim, Germany) at 75 °C. Following addition of 1 ml 0.4 M barium acetate (Sigma-Aldrich, Steinheim, Germany) and 100 μl 4.5 mM glucotropaeolin (Phytolab, Vestenbergsgrueht, Germany) as an internal standard, the samples were stirred lightly, heated for 5 min at 75 °C, and centrifuged at 12,000 rpm for 10 min. The resulting supernatants were collected, and the pellets extracted two more times with 6 ml 70% methanol at 75 °C, stirred, and centrifuged. The supernatants were pooled, and added to 25 ml 70% methanol; 6 ml was applied to a 500 μl DEA-Sephadex A-25 ion-exchanger (acetic acid-activated, Pharmacia, Uppsala, Sweden), and washed with 10 ml bi-distilled (Millipore) water. Subsequently, 2 ml 0.02 M acetate buffer and 250 μl purified aryl sulfatase (Sigma-Aldrich, Steinheim, Germany) was applied, and left for 16 h before the desulfo-compounds were eluted with 4 ml bi-distilled water, and filtered through a 0.45 μm Q-Max nylon filter from Frisenette ApS (Knebel, Denmark). Desulfo-glucosinolates were determined by high-performance liquid chromatography on a Dionex Ultimate HPLC 3000 system from Dionex (Germering, Germany) using a Merck LiChroCART Purospher STAR RP-18e column (5 μm, 250 × 4.6 mm), and a 50 μl sample volume. From 0 to 1 min, the mobile phase was 99% purified water and 1% acetonitrile (20%, Th. Geyer, Renningen, Germany). From this point forward, a gradient of 1–99% acetonitrile in
purified water was selected from 1 to 21 min followed by 10 min with 99% acetonitrile. The flow rate was 1 ml min⁻¹ during the determination, and was run at a 229 nm wavelength. Each individual GLS amount present in the sample was calculated by means of the internal standard (glucotropaeolin), and corrected for relative response factor (The Commission of the European Communities, 1990).

2.4. Statistical analysis

All statistical analyses were conducted in SAS (SAS Institute Inc., Cary, NC, release 9.2, 2000). The fertilizer treatment combinations were considered individual factors and statistical contrasts (general linear model) were determined to test significant differences (p < 0.05). Data were expressed as means (n = 4 ± standard error (Figs. 1 and 2). All data were tested with Tukey’s (1949) one degree of freedom test for interaction. The following variables required a logarithmic transformation prior to analyses due to the lack of homogeneity of variances: N/S ratio tested 40 DAT, and total GLS concentration in Experiment I; and the indole/aliphatic ratio 54 DAT in Experiment II. The N/S ratio in Experiment I was analyzed with contrasts in a general linear mixed model, due to a greater S₀ treatment variance compared to S₀.75 and S₁.5, and consequently the data could not be transformed. The relationships between N/S ratio or soil NH₄⁺/NO₃⁻ ratio and aliphatic, indole, and total GLSs were investigated using a simple linear regression.

3. Results

3.1. Biomass and plant soil N and S concentrations

Experiment I resulted in average total aboveground biomass of 17.56 and 27.56 g DW pot⁻¹ at 40 and 54 DAT, respectively, which was significantly affected by the S treatment at 54 DAT (p < 0.01) (Fig. 1, 40 DAT not shown). Furthermore, an N and S treatment interaction was observed at 54 DAT. The N_split generated the highest g DW pot⁻¹ biomass under the S₀ treatment, whereas N_non-split under the S₀.75 treatment was highest. The N_split exhibited increased biomass for S₀ compared to S₀.75 and S₁.5 treatments. Total aboveground biomass for N_non-split showed increased g DW pot⁻¹ for S₀.75 compared to S₀ and S₁.5. Experiment II exhibited no significant aboveground biomass effects of splitting the S dose (data not shown).

The N and S concentrations were significantly affected by the N and S treatments (Fig. 2, Experiment I). The N_split under S₀.75 and S₁.5 treatments showed a higher concentration of N compared to N_non-split. The S treatment exhibited no influence on N concentration. Cabbage plant S concentration increased concomitantly with increased levels of applied S in the N_split treatment. Similar results were obtained under S₀ and S₀.75 treatments for N_non-split. Splitting N increased the S concentration under the S₁.5 treatment. Splitting S and N doses in Experiment II did not yield significant results (data not shown). Experiment I results showed that the N/S ratio decreased significantly with increasing S treatments in both N treatments (Table 3). A difference in N/S ratio was detected between the N_split and N_non-split treatments under the S₀.75 treatment. In Experiment II, S_split and S_non-split had no effect on the N/S ratio (Table 4).

Soil samples from early harvested plants (40 DAT) indicated higher NO₃⁻ and total inorganic N concentrations from the N_split treatment (Table 1). In Experiment I (Table 1), the soil NH₄⁺ and total inorganic N concentration was higher when the N dose was not split compared to split in all S treatments at final harvest (54 DAT). Under N_split, the NO₃⁻ concentration was higher than in N_non-split. An increase in S treatment resulted in a rise in the total inorganic soil N concentration. Soil S concentration increased with increased S levels, but was not affected by N treatments. In Experiment II, S treatments only affected S concentration, and N treatments exhibited the same pattern as in Experiment I (Table 1).

3.2. Glucosinolate concentrations

The GLSs identified included glucoiberin (3-methylsulphinylpropyl GLS), progoitrin ((R)-2-hydroxy-3-butenyl GLS), glucoraphanin (4-methylsulphinylbutyl GLS), sinigrin (2-propenyl GLS), gluconapin (3-butenyl GLS), 4-hydroxy glucoiberin (4-hydroxy-3-indolylmethyl GLS), glucobrassicin
Table 1
Inorganic N and S (mg kg⁻¹) soil concentrations at the early sampling (40 DAT, n = 2), and in Experiments I and II at final harvest (54 DAT, n = 4).

<table>
<thead>
<tr>
<th>Nutrient supply</th>
<th>Experiment I (54 DAT)</th>
<th>Nutrient supply</th>
<th>Experiment II (54 DAT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>NO₃⁻</td>
<td>Total N₄₅.mouse</td>
</tr>
<tr>
<td>Nₘ₈</td>
<td>96</td>
<td>148</td>
<td>244</td>
</tr>
<tr>
<td>Nₘ₈₀/₀</td>
<td>10</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td>Significance</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

N: Nitrogen, S: Sulfur; * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

a Different lower case letters indicate significant differences (p ≤ 0.05) between means (n = 4) of S treatments for the same N treatment. Different upper case letters indicate significant differences (p ≤ 0.05) between means (n = 4) of N treatments for the same S treatment (statistical contrasts).

b Different lower case letters indicate significant differences (p ≤ 0.05) between means (n = 4) of N treatments for the same S treatment. Different upper case letters indicate significant differences (p ≤ 0.05) between means (n = 4) of S treatments for the same N treatment (statistical contrasts).

(3-indolymethyl GLS), and 4-methoxy glucobrassicin (4-methoxy-3-indolymethyl GLS). The most abundant were sinigrin and glucobrassicin, with a maximum of 2213 and 4189 μg g⁻¹ DW, respectively. Splitting the N dose resulted in a higher indole/alphatic ratio before the 2nd treatment was initiated (40 DAT, Table 2). Aliphatic, indole, and total GLSs 40 DAT were not significantly different between Nₘ₈ and Nₘ₈₀ treatments.

At 54 DAT, splitting the N treatment in Experiment I resulted in a higher indole concentration (68%) and total GLSs (26%) compared to Nₘ₈₀, when plants were administered the highest S levels (Table 3). The aliphatic GLS concentration under the Nₘ₈₀(split treatment was highest when plants were administered S₀ (87%) or S₁₅ (27%) compared to equal S levels under the Nₘ₈ treatment. Increasing S levels from 0 to 0.75 g pot⁻¹ increased aliphatic, indole, and total GLSs concentrations when the N dose was split. Increasing the S dose did not affect indole or total GLSs in the Nₘ₈₀ treatment. The indole/alphatic ratio was higher for Nₘ₈ split compared with Nₘ₈₀ at each S level. Each individual GLS concentration, with the exception of 4-hydroxy glucobrassicin and glucanip in increased S levels (from 0 to 1.5 g S pot⁻¹) under the Nₘ₈₀ treatment (Table 3). In the Nₘ₈₀ treatment, m. progoitrin, sinigrin, and glucanip concentrations reached a higher level in S₀ and S₁₅ compared to S₀.75. No clear pattern emerged in individual GLS response to the three different S levels under Nₘ₈ and Nₘ₈₀ treatments.

The timed S fertilizer treatment in Experiment II exhibited no effect on alphatic GLSs (Table 4). On the other hand, Nₘ₈₀ treatment generated an increased alphatic GLS concentration of Nₘ₈ for Sₘ₈ and Sₘ₈₀ treatments. The Nₘ₈₀ treatment showed a higher indole GLS concentration under Sₘ₈₀ treatment compared to Sₘ₈: furthermore, in the Nₘ₈ treatment, Nₘ₈₀ exhibited a higher concentration relative to Nₘ₈₀ treatment. The total GLS concentration showed the same pattern as indole GLSs, except N had

Table 2
The early sampling effects of Nₘ₈ and Nₘ₈₀ and Sₘ₈ and Sₘ₈₀ on N/S ratio, aliphatic, indole, and total glucosinolate concentrations and indole/alphatic ratio on white cabbage aboveground biomass harvested at 40 DAT.

<table>
<thead>
<tr>
<th>Nutrient supply</th>
<th>N/S ratio</th>
<th>Glucosinolates (μg g⁻¹ DW)</th>
<th>Indole/alphatic ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aliphatic</td>
<td>Indole</td>
</tr>
<tr>
<td>Nₘ₈</td>
<td>11.18</td>
<td>1526</td>
<td>5050</td>
</tr>
<tr>
<td>Sₘ₈</td>
<td>8.29</td>
<td>1570</td>
<td>5380</td>
</tr>
<tr>
<td>Nₘ₈₀</td>
<td>8.15</td>
<td>1908</td>
<td>4424</td>
</tr>
<tr>
<td>Sₘ₈₀</td>
<td>8.43</td>
<td>1810</td>
<td>3883</td>
</tr>
</tbody>
</table>

Significance

| S | NS*          | NS          | NS      | NS      | NS      |
| N | NS          | NS          | NS      | NS      | NS      |
| N x S | NS          | NS          | NS      | NS      | NS      |

N: Nitrogen, S: Sulfur; ** p ≤ 0.01.

* Different lower case letters indicate significant differences (p ≤ 0.05) between means (n = 4) of S treatments for the same N treatment. Different upper case letters indicate significant differences (p ≤ 0.05) between means (n = 4) of N treatments for the same S treatment (statistical contrasts).

* NS: not significant.
**Table 3**
Experiment I. The \( N_{\text{split}} \) and \( N_{\text{non-split}} \) and \( S_0, S_0/2 \) and \( S_0 \) effects on \( N/S \) and indole/aliphatic ratios, and glucosinolate concentrations in white cabbage aboveground biomass harvested at 54 DAT.

<table>
<thead>
<tr>
<th>Nutrient supply</th>
<th>N/S ratio</th>
<th>Glucosinolates* (^{\text{µg} \cdot \text{g}^{-1} \text{DW}})</th>
<th>N/S ratio</th>
<th>Indole/aliphatic ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GLI</td>
<td>PRO</td>
<td>GLR</td>
</tr>
<tr>
<td>( N_{\text{split}} )</td>
<td>( S_0 )</td>
<td>13.40 a M^f</td>
<td>294 b N</td>
<td>39 b N</td>
</tr>
<tr>
<td>( S_0/2 )</td>
<td>9.84 b P</td>
<td>358 a P</td>
<td>55 a P</td>
<td>16 b P</td>
</tr>
<tr>
<td>( S_0 )</td>
<td>9.14 c X</td>
<td>414 a X</td>
<td>59 a Y</td>
<td>26 a X</td>
</tr>
<tr>
<td>( N_{\text{non-split}} )</td>
<td>( S_0 )</td>
<td>12.13 a M</td>
<td>425 a M</td>
<td>92 a M</td>
</tr>
<tr>
<td>( S_0/2 )</td>
<td>8.93 b Q</td>
<td>406 a P</td>
<td>66 a P</td>
<td>17 a P</td>
</tr>
<tr>
<td>( S_0 )</td>
<td>9.26 b X</td>
<td>471 a X</td>
<td>87 a X</td>
<td>20 a X</td>
</tr>
</tbody>
</table>

Significance

- S: ** ** NS NS *** NS *** NS ** NS NS NS NS NS NS NS NS NS
- N: NS NS NS NS NS ** NS NS NS NS NS NS NS NS NS NS NS
- N x S: NS NS NS NS ** NS NS NS NS NS NS NS NS NS NS NS NS

\( N \): Nitrogen; \( S \): Sulfur; ^*^ \( p < 0.05 \), ^**^ \( p = 0.01 \), ^***^ \( p < 0.001 \).


\(^b\) Different lower case letters indicate significant differences \( (p < 0.05) \) between means \( (n = 4) \) of \( S \) treatments for the same \( N \) treatment. Different upper case letters indicate significant differences \( (p < 0.05) \) between means \( (n = 4) \) of \( N \) treatments for the same \( S \) treatment (statistical contrasts).

\(^c\) NS: not significant.

**Table 4**
Experiment II. The \( S_{\text{split}} \) and \( S_{\text{non-split}}, N_{\text{split}} \) and \( N_{\text{non-split}} \) effects on \( N/S \) and indole/aliphatic ratios, and glucosinolate concentrations on white cabbage aboveground biomass harvested 54 DAT.

<table>
<thead>
<tr>
<th>Nutrient supply</th>
<th>N/S ratio</th>
<th>Glucosinolates* (^{\text{µg} \cdot \text{g}^{-1} \text{DW}})</th>
<th>N/S ratio</th>
<th>Indole/aliphatic ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GLI</td>
<td>PRO</td>
<td>GLR</td>
</tr>
<tr>
<td>( S_{\text{split}} )</td>
<td>( N_{\text{split}} )</td>
<td>8.27</td>
<td>397</td>
<td>54</td>
</tr>
<tr>
<td>( S_{\text{non-split}} )</td>
<td>9.14</td>
<td>476</td>
<td>86</td>
<td>23 a X</td>
</tr>
<tr>
<td>( S_{\text{split}} )</td>
<td>9.63</td>
<td>414</td>
<td>59</td>
<td>26 a M</td>
</tr>
<tr>
<td>( S_{\text{non-split}} )</td>
<td>9.26</td>
<td>471</td>
<td>87</td>
<td>20 a X</td>
</tr>
</tbody>
</table>

Significance

- S: NS NS NS NS NS NS NS NS NS NS NS NS NS NS NS NS NS
- N: NS NS NS NS NS ** NS NS NS NS NS NS NS NS NS NS NS
- N x S: NS NS NS NS ** NS NS NS NS NS NS NS NS NS NS NS NS

\( N \): Nitrogen; \( S \): Sulfur; ^*^ \( p < 0.05 \), ^**^ \( p < 0.01 \).

\(^a\) For abbreviations please consult Table 3.

\(^b\) Different lower case letters indicate significant differences \( (p < 0.05) \) between means \( (n = 4) \) of \( N \) treatments for the same \( S \) treatment. Different upper case letters indicate significant differences \( (p < 0.05) \) between means \( (n = 4) \) of \( S \) treatments for the same \( N \) treatment (statistical contrasts).

\(^c\) NS: not significant.
Fig. 3. The relationship between aliphatic (black circle), indole (white circle), and total (black triangle) GLSs, and soil NH$_4^{+}$/NO$_3^-$ ratio 54 days after transplantation (DAT) in Experiments I and II. Aliphatic GLS = $175 \times + 1885$, $R^2 = 0.28$ ($p = 0.0024$). No relationship was found for indole ($p = 0.0535$) or total GLSs ($p = 0.7016$). Dashed lines are 95% confidence bands.

no overall significant effect. Splitting the N fertilization treatment resulted in lower concentrations in three out of eight individual GLSs (glucoraphanin, 4-hydroxy glucobrassicin, and 4-methoxy glucobrassicin), as long as the N treatment was not split (Table 4). For these three GLSs plus sinigrin, the concentrations were higher when N was not split compared to N$_{split}$ under the S$_{split}$ treatment. Sinigrin and glucoraphanin produced higher concentrations under S$_{non-split}$ in the N$_{non-split}$ treatment in contrast to 4-hydroxy glucobrassicin, glucobrassicin, and 4-methoxy glucobrassicin, which resulted in higher concentrations in N$_{split}$.

3.3. Soil NH$_4^{+}$/NO$_3^-$ ratio and glucosinolate relationships

The aliphatic GLS concentrations showed a linear relationship with soil NH$_4^{+}$/NO$_3^-$ ratio in Experiment I and II ($R^2 = 0.28$, $p = 0.0024$, Fig. 3). Indole and total GLSs did not exhibit this relationship.

3.4. N/S ratio and glucosinolate relationships

The relationship between N/S ratio and aliphatic, indole, and total GLSs in Experiment I is depicted in Fig. 4. The N$_{split}$ treatment (Fig. 4 A) exhibited a significant linear relationship between the aliphatic ($R^2 = 0.59$, $p = 0.0035$), indole ($R^2 = 0.55$, $p = 0.0055$), and total GLSs ($R^2 = 0.62$, $p = 0.0025$) and the N/S ratio; whereas no significant relationship was detected with the N$_{non-split}$ treatment (Fig. 4 B). In Experiment II, no relationship between the N/S ratio and the GLS groups was observed (data not shown).

4. Discussion

4.1. Split versus non-split N treatments

Different timed N fertilizer treatments had a significant effect on GLS concentration and composition. A split N treatment in Experiment I resulted in increased indole GLS concentration (Table 3). Consequently, this treatment provided enhanced supply of these nutrients for indole GLS biosynthesis. The synthesis of indole GLSs requires two N atoms relative to one N atom required in aliphatic GLS synthesis, which might explain the requirement for enhanced nutrient supply (Mithen, 2001). These results are congruent with the N$_{non-split}$ treatment, which produced higher aliphatic GLS concentrations. In addition, N$_{non-split}$ was consistent with low N treatment until 40 DAT when the N$_{non-split}$ dose was administered. Stavridou et al. (2012) and Schonhof et al. (2007) reported similar aliphatic GLS increases in broccoli under low N supply conditions.

The choice of urea as N source meant that the N treatments also differed in the soil NH$_4^{+}$/NO$_3^-$ ratio. In the N$_{split}$ treatment nitrification of NH$_4^{+}$ to NO$_3^-$ had proceeded further than in N$_{non-split}$ at the final harvest, resulting in a 74% NH$_4^{+}$ and 26% NO$_3^-$ average in N$_{non-split}$ compared to 33% NH$_4^{+}$ and 67% NO$_3^-$ average in N$_{split}$ (Table 1). Former studies in Brassicaceae grown hydroponically showed increased aliphatic, indole, and total GLSs consistent with an increased NH$_4^{+}$/NO$_3^-$ ratio (Fallow et al., 2011; Kim et al., 2006). This is in part congruent with our results, which showed a linear relationship between higher soil NH$_4^{+}$/NO$_3^-$ ratio and increased aliphatic GLSs (Fig. 3). However, indole and total GLSs did not respond likewise under the same conditions (Fig. 3). Our results should, however, be seen in light of a fluctuating NH$_4^{+}$/NO$_3^-$ ratio dependent on treatment, and the high NH$_4^{+}$ level occurred relatively late in the growing period under the N$_{non-split}$ treatment. Finally, a lower NH$_4^{+}$ level was observed early and late in the growing period for the N$_{split}$ treatment (Table 1). These fluctuating NH$_4^{+}$/NO$_3^-$ ratios were similar to those expected after application of organic fertilizers, e.g., in organic production systems (Dambreville et al., 2008).

The indole/aliphatic ratio was doubled when the N dose was split due to increased indole GLSs, and decreased aliphatic GLS
concentrations. Brown et al. (2002) suggested that differences in indole GLS concentrations were more susceptible to environmental factors, whereas variability in aliphatic GLSs was due to genetic variation among species or cultivars. Following the results of our study, the environmental effects of N treatment on aliphatic GLSs must still be considered, as neither environmental nor genetic variation is solely responsible for GLS changes. The indole and aliphatic GLS ratio is important, because the different GLS groups exhibit different health qualities and benefits. In a review, Latte et al. (2011) suggested that the degradation product of the aliphatic GLS glucoraphanin, isoctiocyamine, might be beneficial to reduce carcinogen activation, whereas the indole GLS neoglucobrassicin should be down regulated. Furthermore from the aliphatic GLS group, the progoitrin nitrites were suggested to protect against colon cancer tumor formation and the sinigrin isoctiocyamine to inhibit metastasis (Barrett et al., 1998; Hwang and Lee, 2006). Both beneficial and harmful effects have been detected among the GLSs and their breakdown products in both of the mentioned GLS groups (Higdon et al., 2007).

4.2. Sulfur effects in split versus non-split N treatments

Glucosinolate composition was affected by increasing S treatments under N<sub>split</sub> and N<sub>non-split</sub> treatments, and showed significant concentration differences among glucoberin, progoitrin, sinigrin, glucoraphanin, and glucobrassicin as well as aliphatic and indole groups, and total GLSs. The plant response to S treatment for the indole GLS glucobrassicin biosynthesis was a higher concentration when N availability was equal during the growth period. Closely connected pathways for N and S assimilation might explain this relationship, where O-acetylseryne plays a decisive role, since O-acetylseryne formation is dependent on N and carbon, and O-acetylseryne contributes to modulation of the assimilatory SO<sub>4</sub><sup>2-</sup>-reduction pathway (Koprivova et al., 2000; Reuveny et al., 1980). In addition O-acetylseryne together with sulfide (S<sub>2</sub>−) form cysteine, a precursor to methionine, one of the amino acid precursor to aliphatic GLSs (Koprivova et al., 2000). At low S availability, O-acetylseryne is accumulated and cysteine levels decrease (Nikiforova et al., 2003), while at low N availability, O-acetylseryne is reduced (Leustek et al., 2000). Therefore, O-acetylseryne accumulation might occur with high N and low S treatments, and consequently result in reduced cysteine, methionine, and ultimately decreased aliphatic GLS synthesis as suggested by Fallovo et al. (2011). This is consistent with our findings if we regard the N<sub>split</sub> treatment as a high N treatment until 40 DAT as discussed in Section 4.1. However, results did not show a notable difference between N/S ratios of the N and S treatments (split and non-split), which should in part be reflected in the aliphatic GLS concentrations, based on the above results. Therefore, S effects on GLS biosynthesis were dependent on N treatment, and might be associated with the different N forms the plants experienced during growth. Nitrogen deficiency in Arabidopsis resulted in decreased adenosine 5'-phosphosulfate (APS) sulfotransferase activity, and enzymatic activity was restored in roots more effectively with NH<sub>4</sub><sup>+</sup> compared to NO<sub>3</sub>− application (Koprivova et al., 2000). The same study demonstrated that the closer related the N source was metabolically to O-acetylseryne, the greater the impact on SO<sub>4</sub>− incorporation. Providing Arabidopsis systemic NH<sub>4</sub><sup>+</sup> led to higher leaf cysteine concentrations compared to NO<sub>3</sub>− (Koprivova et al., 2000). These are results which consisted with our N<sub>non-split</sub> treatment, where only 1.8 g N pot−1 were applied until 40 DAT, and consequently relatively high NH<sub>4</sub><sup>+</sup> soil concentrations were measured after the final harvest (Table 1). Increased NH<sub>4</sub><sup>+</sup> soil concentrations resulted in higher aliphatic GLS concentrations, which are consistent with an increased cysteine concentration, similar to the Arabidopsis study. Increased APS sulfotransferase activity from NH<sub>4</sub><sup>+</sup> rather than NO<sub>3</sub>− also heightened S assimilation. This might have caused a methionine formation increase, and contributed to enhanced aliphatic GLS concentration in the N<sub>non-split</sub> treatment (Brunold, 1993). A similar trend was reported in broccoli, where increasing the S application increased aliphatic and indole GLSs, when the N application was high (Schonhof et al., 2007). To our knowledge, this current report is the first demonstrating that timing alone, and not N fertilizer application dose, played a role in plant response to S, and the consequent amount and composition of GLSs produced in plants. These results show promise in N management for crop production to alter GLS composition in cabbage and possibly the health value aspects.

The effects of the N and S treatments were not detected in the aliphatic, indole, and total GLS concentrations at early samplings (40 DAT) (Table 2). However, the indole/aliphatic ratio differed between N treatments. These results indicated that differentiation between indole and aliphatic GLSs was in progress before the 2nd treatment, although it was not reflected in the GLS groups.

In general, increasing the S application resulted in increased individual aliphatic, indole, and total GLS concentrations (Table 3), which was congruent with our expectations; glucosinolates are S-containing molecules, and previous studies have demonstrated the same trend (Omirou et al., 2009; Rosen et al., 2005; Stavrídou et al., 2012). Higher GLS concentrations were also reflected in increased plant S concentration in the N<sub>split</sub> treatment (Fig. 2). Overall, the N and S fertilizer treatments significantly affected plant N and S concentrations, respectively. We observed the absence of an interaction between the two fertilizer treatments, in contrast to studies on broccoli and white cabbage, where an interaction between N and S was reported concomitant to plant S concentrations (Omirou et al., 2009; Rosen et al., 2005). However, in the present study an interaction between N and S affected total aboveground biomass and GLS concentration, but the effects of N and S were equivocal. We suggest a possible relationship between total GLS concentration and aboveground biomass, since in the N<sub>split</sub> treatment the lowest total GLS concentration was found in the S treatment producing the largest biomass and vice versa, which could reflect a dilution or concentration effect, respectively (Fig. 1, Table 3).

4.3. Split versus non-split S treatments

Similar to N treatments, plants exhibited variable responses to split versus non-split S doses, and responses were dependent on N treatment (Table 4). An initial 2.4 mg S kg−1 soil concentration was measured, a level considered adequate for cabbage production. Therefore, S was not applied with the initial N given 6 DAT. In Experiment I, and Experiment II S<sub>non-split</sub> treatment, plants might have experienced S deficits midway through the experiment, as S was not provided until 40 DAT. This might explain increased indole GLS concentrations under S<sub>non-split</sub> treatment in Experiment II. Similarly, S deficiencies in Arabidopsis thaliana resulted in increased tryptophan levels, an amino acid precursor of the indole GLS group (Fahey et al., 2001; Nikiforova et al., 2003). In addition, former studies indicated aliphatic GLSs were more S-demanding than indole GLSs, with methionine (among others) as a precursor, which is an S-containing amino acid (Fahey et al., 2001; Omirou et al., 2009). However, increased S demand in the aliphatic GLS group was not reflected in S<sub>split</sub> and S<sub>non-split</sub>, but only in N<sub>split</sub> and N<sub>non-split</sub> treatments, where enhanced S supply resulted in increased aliphatic GLS group concentrations in Experiment I. However, quantitative analyses showed that the N treatments exhibited the overall effects on aliphatic GLS concentrations. It is interesting that the S<sub>split</sub> and N<sub>split</sub> combination did not result in a higher GLS concentration than S<sub>non-split</sub>, since splitting both nutrients during plant growth should have provided equal nutrient availability. We
propose that although nutrients were presumably more equally available to the plants when N and S was split, the nutrient soil ratio, and thereby the nutrient ratio in the plant was not optimal with respect to GLS synthesis. There is no doubt that the plant S status affects the biosynthesis of GLSs and that the enzymatic control of sulfate partitioning in either primary (APS reductase) or secondary (APS kinase) metabolism is closely balanced but that the end products are under more complex control (Falk et al., 2007; Mugford et al., 2011). This complexity is underlined by studies done on myeloblastosis (MYB) transcription factors, regulating some of the GLS biosynthesis genes in Arabidopsis, which have shown that there is no clear reflection in the transcript level of biosynthetic genes in planta, and the GLS accumulation (Sanderby et al., 2010).

4.4. N/S ratio and glucosinolate relationships

Plant N:S ratio differed most notably with respect to S treatment. The highest total GLS concentrations were obtained with a N:S ratio between 9.14 and 9.63 in Experiments I and II; in addition results showed decreased total GLSs with an increased N:S ratio (Tables 3 and 4). In broccoli and kohlrabi (B. oleracea L. var. gongyloides), a close association was reported between the N:S ratio and decreased GLS concentration, or the glucosinolate breakdown products, isothiocyanates, when the N:S ratio was higher than 10:1, consistent with our results (Gerendas et al., 2008; Schonhof et al., 2007). The significant linear relationship between N:S ratio and GLS concentration in the Nsplit treatment, compared to the absence of a relationship under the Nnon-split treatment emphasizes that treatments exhibited varied effects on plant N and S concentrations and GLS biosynthesis (Fig. 4). This indicated that the actual GLS concentration cannot be predicted on the basis of the N:S ratio itself, as we observed notable variation in total GLS concentration despite similar N:S ratios. The N:S ratio will likely provide the highest GLS concentration within specific experimental criteria, and for the selected species, a given significant relationship. Fallovo et al. (2011) reported a linear relationship between N:S ratio and total GLS concentration in two Brassica species, congruent with our results; the study showed the highest GLS concentration at a N:S ratio of 4:1 in Brassica rapa and 6:1 in Brassica juncea. Recently Omirou et al. (2012) found a non-linear relationship between leaf NO3 −N and relative dry matter production in Eruc a sativa Mills, indicating the critical applied N fertilizer level and NO3 −N in the plant for optimal growth. Furthermore they suggest that these critical levels could be used for optimizing plant GLS content. The different approaches support further investigations on the relationships between the amount and type of N fertilizer and the biosynthesis of GLSs.

The overall results of our study indicated that the timing of N treatment exhibited a great effect on GLS concentration in white cabbage, and the influence of S treatment was dependent on the N fertilizer treatment. The Nsplit treatment increased indole and total GLSs, whereas the Nnon-split treatment resulted in higher aliphatic GLS concentration. Splitting the S treatment had a smaller effect, and only in combination with the Nsplit treatment. Plant N:S ratio exhibited a relationship with the GLS concentration in the Nsplit treatment, indicating not only a species specific but also a treatment dependent relationship. This knowledge shows promise for future cabbage crop management programs to optimize fertilizer programs, and for beneficial health aspects for human consumption.

Acknowledgments

We are grateful to Kristian Kristensen for his valuable assistance with statistical analyses and to Kai Greven for providing feedback during the writing process. This work was a part of The “MaxVeg” project financed by The Danish Council for Strategic Research.

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Nitrogen split dose fertilization, harvest time and frost effects on phytochemical content and sensory properties of curly kale (*Brassica oleracea* L. var. *sabellica* L.)

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Manuscript in preparation for submission to a peer reviewed journal
Abstract

The aim of this study was to investigate how glucosinolate (GLS), flavonoid glycoside, and sugar concentrations in curly kale responded to split dose and reduced fertilization of nitrogen (N) and late season frost. Development of the compounds throughout the cultivation period and effects on sensory properties in combination with N were assessed. Split N dose fertilization decreased aliphatic GLS concentration, whereas reduced N application increased aliphatic and total GLS concentration. Different concentration optimums throughout the cultivation period were revealed; aliphatic and total GLSs and sugar increased, whereas kaempferol and total flavonoid glycosides showed higher concentrations in 13 week old plants. Frost exposure altered sugar composition slightly but not GLS or flavonoid glycoside composition and concentrations. The stability of the chemical composition during frost exposure was reflected in the sensory profile, where the most significant effect of frost related to an alteration in texture. Bitterness, astringency or pungent aroma and flavor, which usually are barriers for cabbage consumption, were not affected by frost exposure. In conclusion, plant age had stronger effects than N fertilizer treatment with respect to alteration of phytochemicals, and frost exposure only caused minor changes in curly kale.

Key words

Glucosinolates; flavonoid glycosides; soluble sugars; split nitrogen dose; frost; sensory evaluation
**Introduction**

Curly kale (*Brassica oleracea* L. var. *sabellica* L.) is an ancient crop which has regained attention due to the increased focus on locally produced food as well as its health-related potential on account of its phytochemical content. It belongs to the *Brassicaceae* family together with i.a. the more widespread broccoli (*B. oleracea* L. var. *italica* L.), cabbages (*B. oleracea* L. var. *capitata* L.), and cauliflower (*B. oleracea* L. var. *botrytis* L.). All varieties are well known for their content of glucosinolates (GLSs) and polyphenols (Björkman et al. 2011, Cartea et al. 2008). The kale crop is normally harvested as leaves in late autumn/early winter in the North European countries (Hagen et al. 2009).

Application of nitrogen (N) fertilizer is essential to both primary and secondary plant metabolism. An optimal ratio between N, carbon (C) and sulfur (S) is thought to exist in the interaction between primary and secondary metabolism (Bryant et al. 1983, Mugford et al. 2011, Scheible et al. 2004). It depends on the phytochemical in question, and the relevant pathway for biosynthesis. Since GLSs contain N, their concentration is suggested to increase when plant growth is restricted by C availability, which shunts excess N to the secondary metabolism. However, GLS structures also rely on C atoms. A study with split dose fertilization of N given to white cabbage showed an increase in total and indole GLS concentration under greenhouse conditions compared with one dose fertilization (Groenbaek and Kristensen 2014). Likewise, limited N is suggested to increase the synthesis of highly C-based phytochemicals such as flavonoid glycosides (Bryant et al. 1983, Scheible et al. 2004). Investigations on curly kale revealed cultivar-dependent responses in GLS and flavonoid glycoside concentrations under decreased N application (Paper III). Lower N application reduced the concentration of several individual GLSs, quercetin and total flavonoid glycosides in contrast to the traditional cultivars, which showed less N responsiveness. The phenylalanine ammonium lyase (PAL) enzyme activity, which is one of three essential steps in the pathway leading to flavonoid biosynthesis, is known to be inhibited by high N levels (Hahlbrock and Grisebach 1979, Ibrahim et al. 2011). Therefore, a more equally distributed N supply throughout the plant growth period obtained with split dose fertilization might affect the primary/secondary balance to a lesser extent than one dose fertilization of N, and consequently the concentration of the potentially health beneficial phytochemicals in kale.

Plant developmental stage has previously proven to influence GLS concentration, as studies on cabbages and kale revealed increases in total GLS concentrations in leaves from the age of eight weeks until 18 weeks (Rosa et al. 1996). More recently, Velasco et al.
(2007) found an optimum of indole and aliphatic GLS concentration in the leaves of kale (acephala group) before the reproductive phase. A study on tronchuda cabbage (B. oleracea L. var. costata DC) showed no differences in phenolic compound composition between five, six and seven month old plants (Sousa et al. 2008). When comparing a moderate level of N fertilization with a high N level no differences were revealed either. Likewise, Schmidt et al. (2010a) found the total flavonoid concentration in six cultivars of curly kale to be unaffected by increase in plant age from three to six months, but with increases in quercetin and isorhamnetin and decrease in kaempferol concentrations. It is a widespread belief that the sensory properties of kale are optimized after plant frost exposure before harvesting for consumers (Hagen et al. 2009, Olsen et al. 2012). The bitter taste of cabbage has been related to GLSs and flavonols, amongst others, and taste is the main influence, more important than health value, when consumers choose food (Cox et al. 2012, Drewnowski and Gomez-Carneros 2000, Pasini et al. 2011, Schonhof et al. 2004). Sugar has also been found to influence the bitter taste, and highly relevant fructose, glucose and sucrose have been identified as a masking agent for the bitter taste of certain GLSs (Beck et al. 2014, Zabaras et al. 2013). As some plants increase their content of cytosolic sugar to prevent ice formation and cell damage when the temperature is below 0 °C, this might be the reason for the assumed improved sensory quality in kale exposed to frost (Strand et al. 2003). Additionally, Hagen et al. (2009) showed that temperatures below 0 °C had a strong negative effect on total flavonol concentration, along with an increase in sugar content. From a cold treatment (0 °C) on rutabaga roots (Brassica napus ssp. rapifera) no changes in the total GLS concentration were found (Shattuck et al. 1991). However, none of these studies included sensory descriptive analysis. The objectives of this study relate to improving the sensory and phytochemical properties of curly kale. They are: (I) to investigate if split dose and reduced fertilization of N and controlled frost treatment change the GLS, flavonoid glycoside and sugar concentrations in curly kale; (II) to study the formation of the compounds mentioned in (I) throughout plant development under different N treatments; and (III) to test if split dose fertilization and reduced supply of N and controlled frost treatment change the texture, aroma and flavor and, if so, to investigate if these changes could be related to phytochemical content.
Materials and methods

Field setup. The field site was located at the Department of Food Science in Aarslev (55°18´N, 10°27´E) on a sandy loam (Typic Agrudalf) soil containing 1.35% organic C, 33% coarse sand, 38.8% fine sand, 12.6% silt, and 13.4% clay. Inorganic N level in the field before planting was 36 kg ha⁻¹. Seeds of the F₁ hybrid kale ‘Reflex’ (SeedCom A/S, Denmark) were sown June 26th and propagated in an unheated greenhouse. Plants at the 4-5 leaf stage were transferred to the field July 24th with a row/plant distance of 50/30 cm, respectively. Main plots of 3×5 m² with the two outermost rows as guard rows to eliminate border effects were arranged in a completely randomized split-plot design with 3 replications (n = 3) per treatment per harvest. Each main plot was divided into 3 sub-plots with two guard rows, one sub-plot per harvest, with one main plot per treatment being reversed with respect to sub-plot order. According to standard practice, 185 kg N ha⁻¹ in total was given in different doses concurrent with the plan outlined in Table 1. Thus, for the 100% N dose, 149 kg N ha⁻¹, applied in addition to the 36 kg N ha⁻¹ already in the soil, amounted to 185 kg N ha⁻¹. The split dose treatments were 2*50% and 3*33% N. They were applied as 2*56.5 kg N ha⁻¹ (2*50% N) on July 24th and August 24th, as well as with 3*25.66 kg N ha⁻¹ (3*33% N) being applied on July 24th, August 24th, and September 26th, respectively (Table 1). Further, treatments with reduced N application were conducted (50% and 33% N) as well as 2*33% N to support the statistics. Granulate calcium ammonium nitrate (NH₄NO₃+Ca, 27% N, Hydro Agri Danmark A/S, Denmark) was used as N source. 30 kg S ha⁻¹ (granulate kieserite, MgSO₄, 20% S, K+S Kali GmbH, Germany) were applied before transplanting. An insect net covered the plots to reduce herbivory until the final harvest. Weeding was done mechanically once and manually once.

Table 1. Scheme of fertilizer treatments and samplings. One dose treatments are 100%, 50%, and 33%. Split dose treatments are 2*50% and 3*33%.

<table>
<thead>
<tr>
<th>Treatment (kg N ha⁻¹) and sampling (x)</th>
<th>100% N</th>
<th>2*50% N</th>
<th>50% N</th>
<th>3*33% N</th>
<th>33% N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planting &amp; fertilize (July 24th)</td>
<td>149</td>
<td>56.5</td>
<td>56.5</td>
<td>25.66</td>
<td>25.66</td>
</tr>
<tr>
<td>Sampling, 8 weeks (August 24th)</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fertilize (August 24th)</td>
<td>0</td>
<td>56.5</td>
<td>0</td>
<td>25.66</td>
<td>0</td>
</tr>
<tr>
<td>Sampling, 13 weeks (September 26th)</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Fertilize (September 26th)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25.66</td>
<td>0</td>
</tr>
<tr>
<td>Sampling, 17 weeks (October 26th)</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>
When the plants were 8 weeks old (August 24th) the first sampling was done immediately before the second application of fertilizer was conducted. Sampling was done as described below. The second sampling was done 13 weeks after sowing on September 26th and the last fertilizer was applied the same day. The final sampling took place on October 26th when the plants were 17 weeks old. During cultivation, day length went from 16 h at planting time in July, 14 h at the August harvest, 11 h at the September harvest to 9 h at final harvest in October. The approximate maximum photosynthetic photon flux density on sunny days was 3800 and 1500 µmol m\(^{-2}\) s\(^{-1}\) at planting and final harvest, respectively. Daily minimum and maximum temperatures during the cultivation period are depicted in Figure 1.

![Figure 1. Daily minimum and maximum temperatures from planting to final harvest, end of July to end of October 2012. Arrows indicate harvest dates. Data was recorded by The Danish Meteorological Institute weather station situated in the experimental fields at AU-Aarslev.](image)

**Plant sampling.** At each harvest the entire above-ground biomass of ten plants from a sub-plot within the main plot was collected and weighted. About 500 g of representative and evenly distributed leaves with a stem width at the widest point of approximately 7 mm were removed from the primary stem of at least three different plants. Leaves were rinsed in cold tap water, chopped finely for 25 s, and approx. 50 g were immediately frozen in liquid nitrogen and stored at -24 °C before freeze-drying in a CHRIST freeze-dryer, Gamma 1-20 (Osterode am Harz, Germany). After freeze-drying, the samples were ground in an Ultra Centrifugal Mill, ZM 200 (Retsch, Haan, Germany). The rest of the leaves
(approx. 200 g) were dried to a constant weight at 80 °C and dry matter (DM) was calculated.

**Frost treatment.** At final harvest, two sets of representative and evenly distributed leaves from the 100%, 2*50%, 50% and 3*33% N treatments were collected as described above. Before chopping, one set of leaves was rinsed for 15 s under cold running tap water followed by 15 s under cold running demineralized water. Entire leaves from each treatment were placed in separate plastic bags with a wet paper napkin to initiate ice formation and the samples were placed in a temperature-controlled freezer for the frost treatment, according to standard practice under investigations of cold hardiness (Pagter et al. 2011). The samples were cooled from 4 °C to –8 °C and back to 4 °C at a rate of 2 °C h⁻¹ and with a two hour treatment at –8 °C. The corresponding set of leaves was kept at 4 °C until sensory evaluation.

**Plant nutrient analyses.** Leaf N and S concentrations were analyzed from the oven dried material. The VDLUFA methods were used to determine plant N and S (VDLUFA 1976a, b).

**Glucosinolate analyses.** The concentration of GLSs was determined as described by Beck et al. (2014).

**Flavonoid glycoside analyses.** Extraction procedures were as described by Scattino et al. (2014) with the modification that total extraction time was 70 min, whereas the analysis was done by HPLC-DAD-MSⁿ as described by Neugart et al. (2012).

**Sugar analyses.** The content of soluble sugars was determined as described by Jensen et al. (Paper IV).

**Sensory profiling.** The sensory profiling was carried out according to Jensen et al. (Paper IV) with the modification that training time was reduced to 2 hours due to the assessor's extensive experience in evaluating kale samples. The list of attributes and their definition is depicted in Table 2. Briefly, samples of 12 g of chopped material from the 100%, 2*50%, 50% and 3*33% N treatments with and without frost treatment were served in transparent plastic trays (ABENA A/S, Aabenraa, Denmark). The matching lids were marked with a unique three-digit number. The panel assessors consisted of 3/7 males/females aged 26 to 58.
Table 2. List of attributes and their definitions.

<table>
<thead>
<tr>
<th>Group</th>
<th>Attribute</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taste/flavor</td>
<td>Sweetness</td>
<td>The basic sweet taste. Sweetness of the juice emitted is to be evaluated after chewing the sample five times.</td>
</tr>
<tr>
<td></td>
<td>Bitter</td>
<td>The basic bitter taste. Evaluated after chewing the sample well.</td>
</tr>
<tr>
<td></td>
<td>Kale</td>
<td>A green taste similar to grass.</td>
</tr>
<tr>
<td></td>
<td>Pungency (mouth)</td>
<td>A prickly sharp sensation in the tissue similar to the one experienced when chewing cress. Evaluated after chewing the samples eight times.</td>
</tr>
<tr>
<td></td>
<td>Astringency</td>
<td>Dry out in the back of the mouth, puckery sensation.</td>
</tr>
<tr>
<td>Texture</td>
<td>Juiciness/Watery</td>
<td>The amount of water released when chewing.</td>
</tr>
<tr>
<td></td>
<td>Crispiness</td>
<td>Crispness, crunchiness at the very first chewing.</td>
</tr>
<tr>
<td>Color</td>
<td>Dark green</td>
<td>Intensity of the dark green color.</td>
</tr>
<tr>
<td>Aroma</td>
<td>Pungent</td>
<td>A prickly sharp sensation in the tissue in the nose similar to the one experienced when evaluating the aroma of cress.</td>
</tr>
<tr>
<td></td>
<td>Hay</td>
<td>Grass dried in the sun. A dry hayloft.</td>
</tr>
<tr>
<td></td>
<td>Resin</td>
<td>Resin aroma.</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>A green aroma similar to grass.</td>
</tr>
<tr>
<td></td>
<td>Cabbage/rape</td>
<td>Fresh cabbage aroma similar to rape before blooming.</td>
</tr>
<tr>
<td></td>
<td>Non-fresh</td>
<td>Intensity of fusty, non-fresh aroma.</td>
</tr>
</tbody>
</table>

**Statistical analyses.** Statistical analyses were done in SAS (SAS Institute Inc., Cary, NC, release 9.2, 2000). The general linear mixed model procedure was used to test least squares means separations ($p \leq 0.05$). PanelCheck v. 1.4.0 (www.PanelCheck.com) was used for evaluation of assessors and for making a 3-way analysis of variance (ANOVA). Pearson’s correlation tests were applied using XLSTAT v. 2013 (Addinsoft, UK).

**Results and brief discussion**

**The effect of split dose fertilization and reduced application of nitrogen on phytochemicals**

The following GLSs were identified: the aliphatic GLSs, including glucoiberin (3-methylsulphinylpropyl GLS), sinigrin (2-propenyl GLS), glucoraphanin (4-methylsulphinylbutyl GLS), gluconapin (3-butenyl GLS), and progoitrin ((R)-2-hydroxy-3-butenyl GLS); the indole GLSs, including glucobrassicin (3-indolymethyl GLS), 4-hydroxy glucobrassicin (4-hydroxy-3-indolymethyl GLS), 4-methoxy glucobrassicin (4-methoxy-3-indolymethyl GLS), and neoglucobrassicin (1-methoxy-3-indolymethyl GLS); and the
aromatic gluconasturtiin (2-phenylethyl GLS). Total GLS concentration was the sum of the analyzed GLSs.

The concentration of aliphatic GLSs was higher in 100% and 2*50% N compared to 3*33% N (17 weeks, Figure 2). This contradicts the findings of Groenbaek et al. (2014), where aliphatic GLS concentrations in white cabbage were higher in a non-split N treatment compared to N split dose under greenhouse conditions. As the GLS structure contains S, a relationship between leaf S concentration and GLS concentration has previously been documented (Schonhof et al. 2007). In the present study a higher leaf S concentration was found in the 3*33% N treatment in 8 week old plants and in the 33% N treatment in 13 week old plants when compared to the other N treatments (Table 3). This was not reflected in increased GLS concentration under the same conditions. Moreover, equal leaf N concentrations among all N treatments in 17 week old plants do not support the suggestions of increased N-based phytochemicals when N availability is sufficient, as the GLS concentrations were not the same among N treatments. The N/S ratio did not differ among N treatments either and could reflect a lacking relationship between N/S ratio and GLS concentrations, otherwise documented in other Brassica varieties previously (Groenbaek and Kristensen 2014, Schonhof et al. 2007).

Reduced N application (33% and 50% N) revealed higher concentrations of aliphatic GLS ($p = 0.0247$) and consequently total GLS ($p = 0.0386$) in 17 week old plants. Reduced N availability at this developmental stage might have shunted excess C to the chain elongation process of methionine in the aliphatic GLSs biosynthesis, as previous studies saw similar increases with decreasing N application (Halkier and Gershenzon 2006, Schonhof et al. 2007, Stavridou et al. 2012).
Figure 2. The effect of non-split (100% nitrogen (N)) and split dose (2*50% and 3*33% N) treatments on aliphatic, indole and total glucosinolate concentrations. An asterisk denotes significant difference ($p \leq 0.05$) among N treatments. Bars are standard error.
Table 3. Effects of plant age (weeks) and nitrogen (N) treatments on plant nutrient concentration (g kg⁻¹ DM) and ratio in curly kale leaves.

<table>
<thead>
<tr>
<th>Plant nutrient</th>
<th>Weeks</th>
<th>100% N</th>
<th>2*50% N</th>
<th>50% N</th>
<th>3*33% N</th>
<th>33% N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf nitrogen</td>
<td>8</td>
<td>62.2 a A</td>
<td>49.6 a B</td>
<td>49.7 a B</td>
<td>46.4 B</td>
<td>46.3 a B</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>43.9 b</td>
<td>32.6 b</td>
<td>30.8 b</td>
<td>40.7</td>
<td>39.0 b</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>40.0 b</td>
<td>37.7 b</td>
<td>30.3 b</td>
<td>38.4</td>
<td>28.9 b</td>
</tr>
<tr>
<td>Leaf sulfur</td>
<td>8</td>
<td>10.2 a C</td>
<td>11.1 a BC</td>
<td>11.1 a C</td>
<td>12.3 a AB</td>
<td>12.3 a AB</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>7.0 b B</td>
<td>7.5 b B</td>
<td>7.0 b B</td>
<td>7.6 b B</td>
<td>9.1 b A</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>7.4 b</td>
<td>7.0 b</td>
<td>6.9 b</td>
<td>7.0 b</td>
<td>6.4 c</td>
</tr>
<tr>
<td>Leaf nitrogen/sulfur ratio</td>
<td>8</td>
<td>6.1 A</td>
<td>4.5 B</td>
<td>4.5 B</td>
<td>3.8 b B</td>
<td>3.8 B</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>6.2 A</td>
<td>4.1 B</td>
<td>4.4 B</td>
<td>5.4 a AB</td>
<td>4.5 B</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>5.4</td>
<td>5.3</td>
<td>4.4</td>
<td>5.5 a</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*Different letters indicate significant differences ($p \leq 0.05$) among plant ages (lower case) and N treatments (upper case, within row) (n = 3) within each flavonoid glycoside group.

For our investigations we selected 18 out of 71 flavonol glycosides (Schmidt et al. 2010b) in kale, including the main flavonoid glycosides and related structures. The investigated flavonoid glycosides are depicted in Table 4. The 3*33% N treatment resulted in higher kaempferol concentration compared to 100% N (17 weeks, Figure 3). The higher concentrations of kaempferol, isorhamnetin, and total flavonoid glycosides as well as monoacylated tri- and tetracylgosides and diacylated tetracylgosides in the 3*33% and 2*50% N treatments in 8 week old plants could be attributed to the initial low N level applied compared to 100% N (Table 4). This was supported by a higher leaf N concentration in 100% N treatment in 8 week old plants when compared to the other N treatments (Table 3). Similarly, Fallovo et al. (2011) reported a negative linear relationship between leaf N concentration and total flavonoid concentration in *B. rapa* and *B. juncea*. The impact of N level is expected, as increased N application was found to reduce PAL activity in the biosynthetic pathway of flavonols and lower the total flavonoid concentration in *Labisia pumila* (Ibrahim et al. 2011). Correspondingly, a relationship between N level and the gene regulation of the phenylpropanoid and flavonoid synthesis was found in arabidopsis (*Arabidopsis thaliana*) (Lea et al. 2007, Scheible et al. 2004).
Table 4. Effects of plant age (weeks) and nitrogen (N) treatments on flavonoid glycoside concentration (mg g\(^{-1}\) DM) in curly kale leaves.

<table>
<thead>
<tr>
<th>Flavonoid glycoside</th>
<th>Weeks</th>
<th>100% N</th>
<th>2*50% N</th>
<th>50% N</th>
<th>3*33% N</th>
<th>33% N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-acylated diglycosides(^a)</td>
<td>8</td>
<td>0.03 b</td>
<td>0.05</td>
<td>0.05</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.09 a A</td>
<td>0.05 B</td>
<td>0.05 B</td>
<td>0.07 AB</td>
<td>0.10 A</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.04 b B</td>
<td>0.04 B</td>
<td>0.04 B</td>
<td>0.06 AB</td>
<td>0.08 A</td>
</tr>
<tr>
<td>Non-acylated triglycosides(^b)</td>
<td>8</td>
<td>0.88</td>
<td>1.19</td>
<td>1.19 b</td>
<td>1.52</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.91 B</td>
<td>1.33 AB</td>
<td>1.82 a A</td>
<td>0.93 B</td>
<td>1.19 B</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.68</td>
<td>0.71</td>
<td>1.18 b</td>
<td>0.92</td>
<td>1.33</td>
</tr>
<tr>
<td>Monoacylated diglycosides(^c)</td>
<td>8</td>
<td>0.08</td>
<td>0.09</td>
<td>0.09</td>
<td>0.11</td>
<td>0.11 b</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.12</td>
<td>0.09</td>
<td>0.08</td>
<td>0.11</td>
<td>0.13 b</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.11 B</td>
<td>0.08 B</td>
<td>0.12 B</td>
<td>0.13 B</td>
<td>0.18 a A</td>
</tr>
<tr>
<td>Monoacylated tri- &amp; tetrangosides(^d)</td>
<td>8</td>
<td>6.15 b B</td>
<td>7.49 A</td>
<td>7.49 b A</td>
<td>8.66 A</td>
<td>8.66 a A</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>8.25 a</td>
<td>8.14</td>
<td>9.19 a</td>
<td>8.05</td>
<td>8.48 a</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>6.91 b</td>
<td>7.18</td>
<td>8.04 b</td>
<td>7.36</td>
<td>7.06 b</td>
</tr>
<tr>
<td>Diacylated tetrangosides(^e)</td>
<td>8</td>
<td>0.10 B</td>
<td>0.13 B</td>
<td>0.13 b B</td>
<td>0.20 a A</td>
<td>0.20 a A</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.10 B</td>
<td>0.12 B</td>
<td>0.21 a A</td>
<td>0.16 ab AB</td>
<td>0.17 ab AB</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.11</td>
<td>0.10</td>
<td>0.15 b</td>
<td>0.12 b</td>
<td>0.15 b</td>
</tr>
</tbody>
</table>

\(^a\)Kaempferol-3-O-sophoroside, Isohamnetin-3-O-D-glucoside-7-O-D-glucoside. \(^b\)Quercetin-3-O-sophoroside-7-O-D-glucoside, Kaempferol-3-O-sophoroside-7-O-D-glucoside, Isohamnetin-3-O-sophoroside-7-O-D-glucoside, Isohamnetin-3-O-triglucoside. \(^c\)Kaempferol-3-O-sinapoyl-sophoroside, Kaempferol-3-O-feruloyl-sophoroside. \(^d\)Kaempferol-3-O-hydroxyferuoyl-sophoroside-7-O-D-glucoside, Quercetin-3-O-sinapoyl-sophoroside-7-O-D-glucoside, Kaempferol-3-O-sinapoyl-sophoroside-7-O-D-glucoside, Kaempferol-3-O-sinapoyl-sophoroside-7-O-D-glucoside, Kaempferol-3-O-feruloyl-sophoroside-7-O-D-glucoside, Quercetin-3-O-sinapoyl-sophoroside-7-O-D-glucoside, Kaempferol-3-O-sinapoyl-sophoroside-7-O-D-glucoside, Kaempferol-3-O-sinapoyl-sophoroside-7-O-D-glucoside, Quercetin-3-O-sinapoyl-sophoroside-7-O-D-glucoside, Kaempferol-3-O-sinapoyl-sophoroside-7-O-D-glucoside, Kaempferol-3-O-sinapoyl-sophoroside-7-O-D-glucoside, Quercetin-3-O-sinapoyl-sophoroside-7-O-D-glucoside, Kaempferol-3-O-sinapoyl-sophoroside-7-O-D-glucoside, Kaempferol-3-O-sinapoyl-sophoroside-7-O-D-glucoside, Kaempferol-3-O-sinapoyl-sophoroside-7-O-D-glucoside. \(^e\)Quercetin-3-O-disinapoyl-triglucoside-7-O-D-glucoside, Kaempferol-3-O-disinapoyl-triglucoside-7-O-D-glucoside.  

\(^f\)Different letters indicate significant differences (p ≤ 0.05) among plant ages (lower case) and N treatments (upper case, within row) (n = 3) within each flavonoid glycoside group.

The effect of low N application (50% and 33% N) resulting in increased flavonoid glycoside concentrations was only consistent throughout the cultivation period for non-acylated and monoacylated diglycosides (Table 4) and kaempferol glycosides (data not included). This almost absent effect of N level on mature plant flavonoid concentrations corresponds with the leaf N concentration, as no differences among N treatments were found at week 17 (Table 3). Monoacylated tri- and tetrangosides, quercetin and total flavonoid glycoside concentrations were previously found to decrease as N application increased in the same
cultivar of curly kale (Paper III). However, N levels were not comparable, as applications went from 90 to 230 kg N ha⁻¹ in the previous study (Paper III).

Figure 3. The effect of non-split (100% nitrogen (N)) and split dose (2*50% and 3*33% N) treatments on kaempferol, quercetin, isorhamnetin and total flavonoid glycoside concentrations. An asterisk denotes significant difference \((p \leq 0.05)\) among N treatments. Bars are standard error.

**The effect of plant developmental stage on phytochemical concentration**

Independent of N treatment the concentration of aliphatic and total GLSs increased in plants aged 8 weeks up to 17 weeks (Figure 4). In contrast, indole GLS concentration dropped in plants older than 8 weeks grown under 100% and 3*33% N treatments.

Similar increases in GLS concentrations were found in kale (acephala group) during vegetative plant development (Rosa et al. 1996, Velasco et al. 2007). Aliphatic GLS concentration in particular accounted for the increase in comparison with this study. Decreasing GLS concentrations after flowering suggest a buildup of GLSs during the vegetative phase as both herbivore detergents and pollinator attractant under the
reproductive stage (Halkier and Gershenzon 2006, Vallejo et al. 2003, Velasco et al. 2007). A relationship between GLS concentration and the degree of pest damage has been suggested (Velasco et al. 2007).

A less complex biosynthesis of the indole GLSs compared to aliphatic GLSs might result in stronger environmental influence on the indole GLSs, as suggested by Brown et al. (2003). According to our results these environmental influences were less pronounced, as only few effects on indole GLS concentration were found, supporting our results as being due to ontogenetic effects rather than responses to differing N treatments.

Figure 4. The effect of plant age (weeks) on aliphatic, indole and total glucosinolate concentration within each nitrogen (N) treatment. Different letters denote significant difference ($p \leq 0.05$) among plant ages for each glucosinolate group. Bars are standard error.
Kaempferol and total flavonoid glycosides (Figure 5) as well as monoacylated tri- and tetracyglycosides (Table 4) had higher concentrations in 13 week old plants grown under 100% and 50% N treatments. Quercetin glycoside concentration decreased from week 8 to 17 irrespective of N treatment but in line with leaf N concentration (Figure 5). Differing responses to N treatments suggest stronger susceptibility of the kaempferol and isorhamnetin glycoside concentrations to N than for the GLS concentration, and therefore also less pronounced ontogenetic effects. In particular, the big difference in N level for the 8 week old plants caused the variation (Figure 3). Conversely, less influence of N on kaempferol and isorhamnetin glycosides was found in curly kale, but under different N levels and in fully grown plants (Paper III).
The development of flavonoid glycosides did not follow the pattern described by Schmidt et al. (2010a) where concentration of quercetin and isorhamnetin aglycones increased whereas kaempferol decreased along the cultivation period, which were associated with lower temperatures and radiation and not plant developmental stage. However, the total flavonoid concentration stayed unchanged during the cultivation period, as in the present study. This is, however, in conflict with the findings of Sousa et al. (2008) who identified a higher level of phenolic compounds in tronchuda cabbage as a result of higher UV light levels at the first harvest (in November).

The effect of frost treatment on phytochemicals and sugar content

Controlled frost treatment of curly kale leaves did not result in differences of the GLS and flavonoid glycoside composition and concentrations (data not included). Fructose content increased whereas sucrose decreased after frost in the N treatments presented in Table 5. The 50% N treatment showed a higher total sugar content after frost compared to the other N treatments.

Table 5. Sugar content (g kg⁻¹ DM) in the leaves of curly kale at different nitrogen (N) treatments, exposed to frost or not.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Sucrose</th>
<th>Total sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% N</td>
<td>0.52 b a</td>
<td>0.13</td>
<td>0.49</td>
<td>1.15 b</td>
</tr>
<tr>
<td>100% N frost</td>
<td>0.45 cd</td>
<td>0.20</td>
<td>0.44</td>
<td>1.09 b</td>
</tr>
<tr>
<td>2*50% N</td>
<td>0.53 ab</td>
<td>0.17</td>
<td>0.51</td>
<td>1.21 b</td>
</tr>
<tr>
<td>2*50% N frost</td>
<td>0.43 d</td>
<td>0.21</td>
<td>0.44</td>
<td>1.08 b</td>
</tr>
<tr>
<td>50% N</td>
<td>0.51 b</td>
<td>0.17</td>
<td>0.52</td>
<td>1.20 b</td>
</tr>
<tr>
<td>50% N frost</td>
<td>0.59 a</td>
<td>0.34</td>
<td>0.54</td>
<td>1.46 a</td>
</tr>
<tr>
<td>3*33% N</td>
<td>0.50 bc</td>
<td>0.08</td>
<td>0.45</td>
<td>1.03 b</td>
</tr>
<tr>
<td>3*33% N frost</td>
<td>0.44 cd</td>
<td>0.14</td>
<td>0.40</td>
<td>0.99 b</td>
</tr>
</tbody>
</table>

Main effects

<table>
<thead>
<tr>
<th>N</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Sucrose</th>
<th>Total sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>0.49</td>
<td>0.17</td>
<td>0.47</td>
<td>1.12</td>
</tr>
<tr>
<td>2*50%</td>
<td>0.48</td>
<td>0.19</td>
<td>0.47</td>
<td>1.14</td>
</tr>
<tr>
<td>50%</td>
<td>0.55</td>
<td>0.25</td>
<td>0.53</td>
<td>1.33</td>
</tr>
<tr>
<td>3*33%</td>
<td>0.47</td>
<td>0.11</td>
<td>0.43</td>
<td>1.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Frost</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Sucrose</th>
<th>Total sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td>no</td>
<td>0.52</td>
<td>0.14 b</td>
<td>0.49 a</td>
<td>1.15</td>
</tr>
<tr>
<td>yes</td>
<td>0.48</td>
<td>0.22 a</td>
<td>0.46 b</td>
<td>1.15</td>
</tr>
</tbody>
</table>

*Different letters show significant difference at \( p \leq 0.05 \).
Together with up-regulated soluble sugar content in the cytosol, antifreeze proteins prevent cell damage in frost tolerant plants (Strand et al. 2003, Theocharis et al. 2012). The increase of fructose and a corresponding decrease of sucrose after frost exposure could be due to sucrose breakdown into glucose and fructose and thereby a lower freezing point, as fructose is smaller and has a higher osmolarity (Table 5). Cold acclimation in frost tolerant plants is normally initiated by day length and low temperatures above 0 °C (Kalberer et al. 2006, Theocharis et al. 2012). In this study, the sugar content was higher in 13 and 17 week old plants compared to those which were 8 weeks old (Table 6). This indicates that the main up-regulation of sugars might have occurred earlier in the season when lower temperatures first occurred and day length became shorter (Figure 1). In cold acclimated kale (acephala group), accumulation of antifreeze proteins resulted in less freezing injuries compared to non-acclimated (Atici and Nalbantoglu 1999). Yet the activity of antifreeze proteins was reported to be low in kale leaves compared to cold acclimated freeze tolerant cereals (Antikainen and Griffith 1997), which indicates that freezing tolerance in curly kale is obtained by several approaches i.a. increased sugar contents or a third factor such as increased flavonoid concentration.

Table 6. Effect of plant age (weeks) on sugar content (g kg\(^{-1}\) DM) of curly kale leaves.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>100% N</th>
<th>2*50% N</th>
<th>50% N</th>
<th>3*33% N</th>
<th>33% N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>8</td>
<td>0.05 b(^a)</td>
<td>0.23 b</td>
<td>0.23 b</td>
<td>0.24 b</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.50 a</td>
<td>0.51 a</td>
<td>0.52 a</td>
<td>0.48 a</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.52 a</td>
<td>0.53 a</td>
<td>0.51 a</td>
<td>0.50 a</td>
</tr>
<tr>
<td>Fructose</td>
<td>8</td>
<td>0.16</td>
<td>0.10</td>
<td>0.10</td>
<td>0.12 b</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.06</td>
<td>0.10</td>
<td>0.06 NT</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.13</td>
<td>0.17</td>
<td>0.17</td>
<td>0.08</td>
</tr>
<tr>
<td>Sucrose</td>
<td>8</td>
<td>0.12 b</td>
<td>0.21 b</td>
<td>0.21 b</td>
<td>0.20 b</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.44 a</td>
<td>0.47 a</td>
<td>0.49 a</td>
<td>0.44 a</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.49 a</td>
<td>0.51 a</td>
<td>0.52 a</td>
<td>0.45 a</td>
</tr>
<tr>
<td>Total sugar</td>
<td>8</td>
<td>0.33 b</td>
<td>0.54 b</td>
<td>0.54 b</td>
<td>0.55 b</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>1.00 a</td>
<td>1.07 a</td>
<td>1.08 a</td>
<td>0.92 a</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>1.15 a</td>
<td>1.21 a</td>
<td>1.20 a</td>
<td>1.03 a</td>
</tr>
</tbody>
</table>

\(^a\)Different letters show significant difference (\(p \leq 0.05\)) among plant ages within sugar species. NT: not detectable.

A study on frost tolerant Rhododendron suggested an increased frost resistance due to high levels of flavonoid glycosides as the glycosides might have the same osmoregulatory effect as soluble sugars, as well as a high number of hydroxyl groups with the ability to form bonds with water, thereby reducing ice formation (Swiderski et al. 2004). Likewise, a
clear increase of flavonoids in cold acclimated arabidopsis was found as compared to non-acclimated (Korn et al. 2008). An increase from week 8 to 13 in kaempferol and total flavonoid glycoside concentrations, similar to sugar content partly support this (Figure 5). However, a subsequent decrease in the flavonoid glycoside concentrations in week 17 and variable results among N treatments contradicts frost resistance effects of flavonoid glycosides in kale. A stable level of flavonol concentration in curly kale after cold storage at 1 °C was reported by Hagen et al. (2009). However, when compared to frost exposure in the field, a 35% drop of total flavonol concentration was revealed. Furthermore, a decrease in sugar content was found when the curly kale was stored, in contrast to an increase in the plants kept in the field. Shattuck et al. (1991) did not find any changes in total GLS level in rutabaga roots stored at 0 °C compared to plants kept under initial growing condition at around 25 °C. But an alteration of the individual GLSs was detected.

*Sensory evaluation of nitrogen and frost treated curly kale*

The sensory evaluation of the 100%, 2*50%, 50% and 3*33% N treatments with and without frost exposure revealed differences between the resin, green and cabbage/rape aromas as well as crispness (Figure 6). GLSs and flavonols have in previous studies been related to bitterness, pungency and astringency (Drewnowski and Gomez-Carneros 2000, Pasini et al. 2011, Schonhof et al. 2004, van Doorn et al. 1998). As the GLS and flavonoid glycoside concentrations showed no differences between frost and non-frost exposure, this corresponded with no observed differences in the attributes connected to GLSs in particular. A correlation test between these attributes, including sweetness, and the phytochemicals analyzed revealed relationships for glucoraphanin, neoglucobrassicin, monoacylated tri- and tetracyglycosides, diacylated tetracyglycosides, and sucrose (Table 7). Interestingly, pungent aroma, bitterness and astringency were negatively related to one or more of the previously mentioned phytochemicals, not supporting previous studies which found positive relationships between GLSs normally considered as bitter: gluconapin, progoitrin, glucobrassicin, and neoglucobrassicin (Pasini et al. 2011, Schonhof et al. 2004, van Doorn et al. 1998).

Regarding the monoacylated tri- and tetracyglycosides and diacylated tetracyglycosides, the relatively high glycoside number might play a masking role of bitterness and astringency, as sugars have been found to mask GLSs and interactions between perception of sweet and bitter are thought to exist (Beck et al. 2014, Schonhof et al. 2004, Zabaras et al. 2013).
Sugar content of kale has earlier been found to be a good predictor of sweet perception, but this was not confirmed in this study (Paper IV). Possibly due to lower sugar contents in the present study compared to the cultivars investigated by Jensen et al. (Paper IV). Furthermore, N level did not result in altered sensory properties in the same study.

Figure 6. Results of the sensory profiling of curly kale leaves grown under different nitrogen (N) and frost treatments. An asterisk denotes significant difference ($p \leq 0.05$) among the treatments

The attributes green and cabbage/rape aroma had higher sensory intensities after frost exposure (Figure 6), which could be due to some degree of cell damage, as the aromas are more likely to be released after tissue damage. However, no reduction in GLS concentration was found. Likewise reduced crispness under the same conditions might be explained by potential cell damage (Figure 6).

In conclusion, the split N dose treatments and frost exposure had less effect on phytochemical composition and concentration. Reduced N level increased aliphatic and total GLS concentrations as well as non-acylated and monoacylated diglycoside and kaempferol glycoside concentrations. Plant age had a strong effect on aliphatic and total GLS concentrations, which increased with plant age. Likewise, quercetin glycosides decreased, whereas kaempferol and total flavonoid glycosides showed an optimum in 13 week old plants. Sugar content increased from week 8 to 13. This means that kale plants
with different sugar contents and phytochemical profiles can be harvested throughout the cultivation period. A potential for reduction of N application without losing phytochemical properties was seen, whereas split N dose did not alter phytochemical composition or concentration.

Frost exposure resulted in fructose content increases and sucrose content decreases, but no changes in phytochemical composition and concentration. The sensory attributes normally considered as barriers for cabbage consumption were not affected by frost. Consequently growers and consumers should not rely on frost to obtain a product with improved sensory or phytochemical properties.

**Acknowledgment**

We are grateful to Astrid Bergmann, Jens Barfod, and Knud Erik Pedersen for assisting during the field experiments, and to Birgitte Foged, Jens Madsen and Antje Bamberg for contributing to the chemical analyses.
Table 7. Correlation between characteristic attributes of curly kale and the components with statistically significant correlations.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Pungent aroma</th>
<th>Sweetness</th>
<th>Bitterness</th>
<th>Pungent flavor</th>
<th>Astringency</th>
<th>GLR</th>
<th>NGLB</th>
<th>MATT</th>
<th>DIT</th>
<th>Sucrose</th>
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<tr>
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<td></td>
<td>0.048</td>
<td>0.029</td>
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<td>0.319</td>
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<td>-</td>
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<tr>
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<td>-</td>
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<td>0.376</td>
<td>0.098</td>
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<td>Astringency</td>
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<td>0.90</td>
<td>-</td>
<td>0.113</td>
<td>0.049</td>
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<td>-</td>
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<td>0.001</td>
<td>0.084</td>
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<td>-</td>
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<td>Sucrose</td>
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<td>-0.72</td>
<td>0.40</td>
<td>0.65</td>
<td>0.73</td>
<td>0.64</td>
<td>-</td>
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</table>

P values are italicized and correlation coefficients (r) are not. Bold indicate significant correlation ($p \leq 0.05$). GLR: Glucoraphanin; NGLB: neo-glucobrassicin; MATT: monoacylated tri- and tetraglycosides; DIT: diacylated tetraglycosides.
Reference list


Pasini, F.; Verardo, V.; Cerretani, L.; Caboni, M. F.; D’Antuono, L. F. Rocket salad (*Diplotaxis* and *Eruca* spp.) sensory analysis and relation with glucosinolate and


This work was a part of the “MaxVeg” project financed by The Danish Council for Strategic Research’s Programme Commission on Health, Food and Welfare.
3.1 Paper II
Chapter 4

4.1 Paper III

Curly kale (*Brassica oleracea* L. var. *sabellica* L.) cultivar selection grown under different nitrogen and sulfur supplies in the field. I. Genetic diversity reflected in growth patterns and phytochemical concentration

Marie Groenbaek, Sidsel Jensen, Susanne Neugart, Monika Schreiner, Ulla Kidmose and Hanne L. Kristensen

Accepted for publication in *Journal of Agricultural and Food Chemistry*, a revised version is in press. Submitted as a joint manuscript with paper IV
Abstract

The objectives of this study were to investigate if genetic diversity between field-grown traditional and F1 hybrid kale cultivars were reflected in different growth patterns and consequently glucosinolate (GLS) and flavonoid glycoside concentration and composition. We evaluated how nitrogen (N) and sulfur (S) supply and biomass allocation modified phytochemicals in two experiments with combinations of three cultivars, four N and two S application levels. Results showed that less growth and higher N concentration in the traditional cultivar ‘Tiara’ was associated with increased indole and total GLSs compared to the traditional ‘Høj Amager Toftø’ and F1 hybrid ‘Reflex’ cultivars, which exhibited higher yield, lower N concentration and different biomass allocation. S application increased total GLS concentration, whereas aliphatic GLS percentage decreased when N application increased. Decrease of six ‘Reflex’ individual GLSs besides the quercetin glycosides and total flavonoid glycosides with increased N indicated higher N responsiveness for ‘Reflex’. In conclusion, differences in cultivar growth patterns were reflected in diverse phytochemical composition.

Keywords

Curly kale; Cultivars; Nitrogen; Sulfur; Glucosinolates; Flavonoid glycosides
**Introduction**

Due to a characteristic phytochemical content, members of the *Brassicaceae* have been of special interest during almost half a century (Fahey et al. 2001). The *Brassica* genus includes several varieties comprising of curly kale (*Brassica oleracea* L. var. *sabellica* L.), red and white cabbage (*B. oleracea* L. var. *capitata* L.), broccoli (*B. oleracea* L. var. *italica* L.), Brussels sprouts (*B. oleracea* L. var. *gemmafera* L.), and cauliflower (*B. oleracea* L. var. *botrytis* L.), all known for the species phytochemical content (Fahey et al. 2001). Kale ancestors can be traced back to landraces and wild kales grown on the Iberian Peninsula and in the Black Sea region (Christensen et al. 2011). Currently, the highly bred cultivars dominate commercially grown fields. However, several traditional cultivars have been studied to identify potential disease resistance, specific agronomic traits, and sensory properties or phytochemicals beneficial to health, such as glucosinolates (GLSs), flavonoids, and carotenoids (Cartea et al. 2008, Kopsell et al. 2007, Schmidt et al. 2010a).

In general, differences in GLS composition and concentration are found in different *B. oleracea* varieties (Cartea et al. 2008, Kushad et al. 1999). Each variety rarely contains more than 12 different GLSs, although more than 120 different GLSs are known (Fahey et al. 2001). Furthermore, GLS composition and concentration differences between cultivars have been identified within cauliflower, white cabbage, broccoli, Brussels sprouts, and kale (Kushad et al. 1999, Nilsson et al. 2006). Some GLS breakdown products have been linked to anti-carcinogenic properties of *Brassica* vegetables (Verhoeven et al. 1997). Moreover, Williams and Pun (2011) associated GLSs with the bitter and pungent taste of cabbages, which was further investigated in the present experiments, and reported by Jensen et al. (2014). Studies suggested different responses in GLS group concentration, e.g. indole and aliphatic are influenced by growing conditions and genetic background, respectively (Brown et al. 2003, Groenbaek and Kristensen 2014). Nitrogen (N) and sulfur (S) fertilizers effect on GLSs have been widely studied, since fertilizers play a major role in GLS biosynthesis, and N and S are incorporated in GLS structure. In brief, several studies reported GLS concentration was generally increased by S fertilizer application in *B. oleracea*; however N level, form, and application timing exhibited diverse responses, possibly due to different responses in primary metabolism induced by genotypic variations in N and S uptake and metabolization (Groenbaek and Kristensen 2014, Jones et al. 2007, Omirou et al. 2009, Rosen et al. 2005, Schonhof et al. 2007, Stavridou et al. 2012). If primary metabolism in relationship to plant growth and protein synthesis is reduced due to limited carbon (C), N, or S, a shift towards secondary metabolism of C-, N-, or S-
containing phytochemicals, such as flavonols and glucosinolates might occur (Bryant et al. 1983, Mugford et al. 2011, Scheible et al. 2004). Consequently, genetic variability between traditional and F1 hybrid cultivars might be reflected in growth patterns, N and S uptake besides plant N and S concentration, biomass allocation to different plant parts, as well as secondary metabolism.

Flavonoid health benefits depend on flavonoid structure (Manach et al. 2005, Zietz et al. 2010). In curly kale, Schmidt et al. (2010b) found 71 flavonoid glycosides based on the aglycones quercetin, kaempferol, and isorhamnetin, and Zietz et al. (2010) reported higher antioxidant activity in quercetin glycosides compared to the corresponding kaempferol glycosides in curly kale. Quercetin, kaempferol, and isorhamnetin aglycones exhibited anti-inflammatory effects (Hämäläinen et al. 2007), whereas, quercetin glycosides showed enhanced absorbance in the human small intestine compared to quercetin aglycone (Hollman et al. 1995). Studies revealed an inverse relationship between N availability and flavonol or total phenolic concentrations in leaf mustard (Brassica juncea), tomato (Lycopersicum esculentum), and arabidopsis (Arabidopsis thaliana) (Li et al. 2008, Stewart et al. 2001). Moreover, a report on eight different field-grown curly kale cultivars showed relatively high flavonoid concentrations in traditional old cultivars in contrast to lower concentrations in hybrids (Schmidt et al. 2010a).

However, the influence of the genetic variation on flavonoid glycoside and GLS concentrations associated to fertilizer strategies, growth pattern and biomass allocation in traditional and F1 hybrid cultivars of curly kale has not been reported. Trials conducted under field growth conditions with less control over biogeochemical environments compared to, for example greenhouse experiments are necessary when testing agronomic practices, particularly when examining a crop stand with relevance to growers and consumers.

Based on testing genetic variation between traditional and F1 hybrid kale cultivars in order to optimize fertilizer management based on genotype, the objectives of this study were: (i) investigate whether GLS and flavonoid glycoside concentration and composition differed markedly among cultivars; (ii) determine if varying N supplies in relationship to growth pattern and biomass allocation modified GLS and flavonoid glycoside composition and concentration; and (iii) investigate field S and N application effects on GLS concentration and composition.
Materials and methods

The field experiment. The field site was located at the Department of Food Science in Aarslev, Denmark (56°18´N, 10°27´E) on a sandy loam (Typic Acrudalf) soil containing 1.35% organic C, 33% coarse sand, 38.8% fine sand, 12.6% silt, and 13.4% clay. Inorganic N and S levels in the field before planting were 10 and < 4 kg ha⁻¹, respectively. Seeds of the F1 hybrid ‘Reflex’ (SeedCom A/S, Denmark), and the traditional cultivars ‘Halvhøj Ekstra Moskruiset Tiara’ (TIARA) accession 1833 and ‘Høj Amager Toftø’ (HAT) accession 4085 (both NordGen germplasm collection (2014)) were sown on 4 April 2011, and propagated in an unheated greenhouse. Plants were transferred to the field on 5 May 2011 with a row/plant distance of 50/60 cm, respectively. Plots of 3×5 m² with the two outermost rows as guard rows to eliminate border effects were arranged in a complete randomized block design with three replication of plots (n = 3) per fertilizer level and cultivar (see below). An insect net was applied over the plots and used for pest management to reduce herbivory until 16 August 2011. Weeding was conducted manually and mechanically once during the experimental trial. The trials included two separate experimental regimes. Experiments were performed as follows: In Experiment I, Reflex, HAT, and TIARA were grown under 30 kg S ha⁻¹ with 90, 135, 185, or 230 kg N ha⁻¹ available in the field (36 plots in total); and under Experiment II, Reflex had either 4 or 30 kg S ha⁻¹ available combined with 90, 135, or 185 kg N ha⁻¹ accessible in the field (18 plots in total). Fertilizer levels applied were adjusted according to the levels in the field measured before planting. S fertilizer was dispersed just prior to planting, and approximately half the total N fertilizer dose was distributed just after planting. The other half was given 6 weeks later. Granulate kieserite (MgSO₄, 20% S, K+S Kali GmbH, Germany) and granulate calcium ammonium nitrate (NH₄NO₃+Ca, 27% N, Hydro Agri Danmark A/S, Denmark) were used as S and N sources, respectively. On 10 October 2011, 10 plants from each replicate plot were harvested, including the entire stalk and leaves (corresponding to 3.1 m²), and total yield was assessed. Four of 10 plants from each plot were evaluated with respect to stalk height from plant base at soil surface to the plant top. Yield parameters were estimated as follows: edible yield as usable leaf weight (stem diameter < 7 mm), and stalk weight without leaves, stems, and top. Plants were maintained at a temperature of 1 °C and 100% relative humidity in black plastic bags until further analysis within 4 days. The described experiments correspond to analysis 2, 3, and 4 reported by Jensen et al. (2014) from which sensory evaluation of the different fertilization approaches were assessed.
Plant analyses. Representative samples of approximately 200 g of separated leaves and stalks from each plot were oven dried at 80 °C to a constant weight, and dry matter content (DM) was calculated. Total leaf and stalk N and S concentrations were analyzed from the oven-dried material. VDLUFA methods were used to determine plant N and S (VDLUFA 1976a, b).

Glucosinolate analysis. Three of the 10 harvested plants per replication plot (n = 3) were randomly selected, and nine evenly distributed leaves per plant with a stem width at the widest point of approximately 7 mm were removed from the primary stem. Curly kale leaves were rinsed in cold tap water, chopped fine, immediately frozen in liquid nitrogen, and stored at -24 °C until freeze-drying in a CHRIST freeze-dryer, Gamma 1-20 (Osterode am Harz, Germany). After freeze-drying, the samples were ground in an Ultra Centrifugal Mill, ZM 200 (Retsch, Haan, Germany). GLS concentration was determined as described by Beck et al. (2014).

Flavonoid glycoside and hydroxycinnamic acid analysis. Twenty mg of the freeze-dried ground leaves were extracted with 600 µL of 60% methanol (MeOH) (Roth, Karlsruhe, Germany), and shaken for 40 min in a Thermomixer™ (Eppendorf™, Thermo Fischer Scientific Inc., Wesseling-Berzdorf, Germany) at 1400 rpm and 20 °C. Hereafter, extracts were centrifuged at 12000 rpm for 10 min. The supernatant was transferred to a new sterile test tube. The procedure was repeated twice with 400 µL 60% MeOH, for 20 min, and 200 µL 60% MeOH for 10 min. The combined supernatants were filtered through a Spin-X (Sigma Aldrich Chemical Co., St. Louis, MO) tube by centrifugation at 3000 rpm for 5 min, and dry evaporated in a vacuum centrifuge. The residue was dissolved in 200 µL of distilled water. Flavonoid glycoside and hydroxycinnamic acid derivative analyses were conducted by HPLC-DAD-MS^n as described by Neugart et al. (2012) for Experiment I and not Experiment II as influences of S on flavonoid glycosides and hydroxycinnamic acid derivatives were not expected.

Statistical analyses. All univariate statistical analyses were performed in SAS (SAS Institute Inc., Cary, NC, release 9.2, 2000). The general linear model and general linear mixed model procedures were applied for comparison of least squares (LS) means (p ≤ 0.05). GLS, flavonoid glycoside, and hydroxycinnamic acid derivative analyses (this study) and for sensory analyses (reported in Jensen et al. (2014)) were made from all treatments in Experiment I except HAT grown under 135 and 185 kg N ha⁻¹. Therefore, each of the ten cultivar and N level combinations in Experiment I were grouped as one factor to compare
chemical data analysis results using statistical contrasts. All linear relationships were investigated using a simple linear regression.

**Results and discussion**

**Growth patterns and glucosinolate concentrations**

**Experiment I: N level and cultivar effects.** The three cultivars showed distinct differences in growth pattern parameters which consisted of total and edible yield, stalk weight and height, leaf and stalk N and S content as well as total N and S uptake. The HAT cultivar exhibited the highest total yield but Reflex generated the highest edible yield (Table 1). The shift in highest total and edible yield between HAT and Reflex respectively, corresponded to stalk data. Likewise higher leaf and stalk N concentrations corresponded to lower yields of TIARA, opposite Reflex. Similar to our findings, Padilla et al. (2007) identified different morphologic and agronomic traits of 148 kale landraces (*acephala* group) resulting in five clusters based on these trait differences. The higher leaf N concentration in TIARA indicated that TIARA growth was not limited by N availability at increased N applications, which was consistent with the capacity of cabbage to take up more N than required for growth (Maynard et al. 1976). Leaf and stalk N did not differ between Reflex and HAT, although HAT edible yield decreased at 230 kg N ha⁻¹, and Reflex showed an increase in yield. Total S uptake increased from 90 to 135 kg N ha⁻¹ applied, whereas total N uptake increased continuously from 90 to 230 kg N ha⁻¹ applied (Table 1).
Table 1. Yield and nutrients in curly kale cultivars (CV) HAT, Reflex, and TIARA grown under four different nitrogen (N) levels (kg ha\(^{-1}\)).

<table>
<thead>
<tr>
<th>N</th>
<th>CV</th>
<th>Total yield(^a)</th>
<th>Edible yield(^a)</th>
<th>Stalk weight(^a)</th>
<th>Stalk height(^b)</th>
<th>Leaf N(^c)</th>
<th>Leaf S(^c)</th>
<th>Stalk N(^c)</th>
<th>Stalk S(^c)</th>
<th>Total N uptake(^d)</th>
<th>Total S uptake(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>HAT</td>
<td>50.1</td>
<td>13.7 d(^e)</td>
<td>19.4 ef</td>
<td>77</td>
<td>23.6</td>
<td>7.7</td>
<td>9.9</td>
<td>3.9</td>
<td>168</td>
<td>57</td>
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<tr>
<td></td>
<td>Reflex</td>
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<td>17.1 bc</td>
<td>19.0 e f</td>
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<tr>
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<td>16.9 f</td>
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<td>27.6</td>
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<td>29.5 b</td>
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<td>22.2 a</td>
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<td>21.3 def</td>
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<td>25.4 bcd</td>
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<td>17.7</td>
<td>5.0</td>
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Main effect

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<th>N</th>
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<th>Edible yield(^a)</th>
<th>Stalk weight(^a)</th>
<th>Stalk height(^b)</th>
<th>Leaf N(^c)</th>
<th>Leaf S(^c)</th>
<th>Stalk N(^c)</th>
<th>Stalk S(^c)</th>
<th>Total N uptake(^d)</th>
<th>Total S uptake(^d)</th>
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<tbody>
<tr>
<td>90</td>
<td>HAT</td>
<td>48.6 c</td>
<td>15.0</td>
<td>18.4</td>
<td>72 c</td>
<td>25.0</td>
<td>7.7</td>
<td>11.5 b</td>
<td>4.6 a</td>
<td>180 c</td>
<td>59 b</td>
</tr>
<tr>
<td>135</td>
<td>HAT</td>
<td>58.0 b</td>
<td>18.1</td>
<td>24.3</td>
<td>83 b</td>
<td>27.1</td>
<td>7.9</td>
<td>13.6 a</td>
<td>4.7 a</td>
<td>236 b</td>
<td>72 a</td>
</tr>
<tr>
<td>185</td>
<td>HAT</td>
<td>64.6 a</td>
<td>19.9</td>
<td>27.1</td>
<td>84 b</td>
<td>26.9</td>
<td>7.4</td>
<td>15.0 a</td>
<td>4.4 ab</td>
<td>254 ab</td>
<td>71 a</td>
</tr>
<tr>
<td>230</td>
<td>HAT</td>
<td>66.8 a</td>
<td>19.3</td>
<td>29.2</td>
<td>92 a</td>
<td>28.3</td>
<td>7.1</td>
<td>15.4 a</td>
<td>4.0 b</td>
<td>278 a</td>
<td>70 a</td>
</tr>
<tr>
<td>CV</td>
<td>HAT</td>
<td>65.8 a</td>
<td>17.9</td>
<td>29.7</td>
<td>93 a</td>
<td>24.7 b</td>
<td>7.1</td>
<td>12.1 b</td>
<td>3.6 c</td>
<td>238</td>
<td>70 ab</td>
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<tr>
<td></td>
<td>Reflex</td>
<td>61.1 b</td>
<td>21.4</td>
<td>23.3</td>
<td>78 b</td>
<td>25.9 b</td>
<td>6.7</td>
<td>11.9 b</td>
<td>4.1 b</td>
<td>229</td>
<td>63 b</td>
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<tr>
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<td>TIARA</td>
<td>51.5 c</td>
<td>14.9</td>
<td>21.2</td>
<td>77 b</td>
<td>29.8 a</td>
<td>8.7</td>
<td>17.5 a</td>
<td>5.6 a</td>
<td>245</td>
<td>72 a</td>
</tr>
</tbody>
</table>

N  | CV  | ***  | ***  | ***  | ***  | NS   | NS   | **  | *    | ***  | *    |
| N  | *** | NS   | *    |     |     |    | NS   | NS  | NS   | NS   | NS   |

\(^{a}\)tonne ha\(^{-1}\), \(^{b}\)cm, \(^{c}\)g kg\(^{-1}\) dry matter, \(^{d}\)kg ha\(^{-1}\). Different letters indicate significant differences (\(p \leq 0.05\)) among cultivar and N level means (\(n = 3\)) within each column.
This result partly supports the close association in plant N and S assimilation, where adenosine 5’-phosphosulfate (APS) plays a special role in relationship to S assimilation (Reuveny et al. 1980) and APS reductase activity, which is controlled by N availability (Koprivova et al. 2000). Possibly *B. oleracea*, with the capacity to accumulate nitrate does not apply strictly to this hypothesis as similar findings of less N and S interaction in relation to plant N and S concentration have been reported in other field studies (Maynard et al. 1976, Rosen et al. 2005, Stavridou et al. 2012). Thus, increasing N application resulted in increased edible yield of Reflex, whereas TIARA showed no response in biomass despite elevated leaf and stalk N concentrations (Table 1). The response of HAT to N was mainly found in increased stalk biomass.

Different growth patterns were associated to a diverse GLS composition observed among the three cultivars. The following GLSs were identified: the aliphatic GLSs, including glucoiberin (3-methylsulphinylpropyl GLS), sinigrin (2-propenyl GLS), glucoraphanin (4-methylsulphinylbutyl GLS), gluconapin (3-butenyl GLS), and progoitrin ((R)-2-hydroxy-3-butenyl GLS); the indole GLSs, including glucobrassicin (3-indolymethyl GLS), 4-hydroxy glucobrassicin (4-hydroxy-3-indolylmethyl GLS), 4-methoxy glucobrassicin (4-methoxy-3-indolylmethyl GLS), and neoglucobrassicin (1-methoxy-3-indolylmethyl GLS); and the aromatic gluconasturtiin (2-phenylethyl GLS). Cultivars differed in total GLS concentration (TIARA 3503 µg g⁻¹ DM, HAT 2799 µg g⁻¹ DM, Reflex 2454 µg g⁻¹ DM, \( p < 0.01 \)). Increased N application resulted in a decreased percentage of the aliphatic GLS group, and a corresponding increase in indole GLS percentage (Figure 1A, \( p < 0.0001 \)). Mithen et al. (2000) indicated a higher demand for N due to indole GLS biosynthesis, which requires 2 N atoms compared to 1 N atom for aliphatic GLS biosynthesis, results which support this study. Studies in broccoli and green cabbage were also congruent with our results, but the present study is the first documented in field-grown curly kale (Jones et al. 2007, Rosen et al. 2005, Stavridou et al. 2012). However, arabidopsis studies found NO₃⁻ addition repressed the shikimate pathway, responsible for synthesis of flavonols and the indole GLS amino acid precursor tryptophan (Scheible et al. 2004, Sønderby et al. 2010b). A negative linear relationship was detected for Reflex, between edible yield and aliphatic or total GLS concentration, respectively (\( p = 0.0193, R^2 = 0.44 \) and \( p = 0.0023, R^2 = 0.62 \), respectively), while TIARA showed a positive linear relationship between indole GLS concentration and edible yield (\( p = 0.0395, R^2 = 0.36 \)).
Suggestions on increased biosynthesis of C-, N-, and S-containing phytochemicals in cases of limited availability to one or more of the compounds have been put forward (Bryant et al. 1983, Mugford et al. 2011, Scheible et al. 2004). For example increased N availability under normal CO2 conditions might increase N-containing phytochemicals such as GLSs as C limits growth. Likewise growth limitation by less N availability might shift to metabolism of phytochemicals containing much C, such as flavonoids. Partly contradicting our results, studies on GLS content of Chinese kale (Brassica alboglabra L.) grown under increasing N levels, showed reduced aliphatic GLS but enhanced indole GLS concentrations with both ambient and elevated CO2 levels (La et al. 2009). In this study the increased indole GLS concentration response in TIARA, reflected limited growth by factors other than N; and to some extent, aliphatic and total GLS decrease in Reflex, where N but not C was presumably scarce. However, GLSs also contain C and the biosynthetic pathways for N- and C-containing phytochemicals cannot be fully distinguished, as indicated above. This was further documented by Sønderby et al. (2010a) who reported an epistatic effect of aliphatic
myeloblastosis (MYB) transcription factor on total indole GLS concentration. Moreover, aliphatic GLSs also contain N, although less than indole GLSs. Intermediate total GLS concentration in HAT, and the absence of a linear relationship with edible yield could be explained by biomass allocation to stalks compared to leaves consistent with increasing N application, not observed in Reflex and TIARA (Table 1). Controlled crop improvement is very often aimed at higher yield, which was demonstrated in our results, where the modern F1 hybrid Reflex showed increased edible yield (Table 1). Therefore, reduced growth and higher N concentration in TIARA might be associated to increased indole GLS percentage, and total GLS concentration, compared with HAT and Reflex, which exhibited higher yield, lower N concentrations, and variable ratios between leaf and stalk growth.

Notable differences between individual GLS concentration of the cultivars were detected (Figure 2). These differences reflected the diverse growing patterns as a result of increasing N application where the homo-methionine derived aliphatic GLSs glucoiberin and sinigrin concentration decreased in Reflex opposite no response in TIARA and HAT. Further cultivar differences were revealed for the dihomo-methionine derived aliphatic GLSs glucoraphanin and gluconapin with concentration decreases in Reflex in contrast to an optimum of glucoraphanin concentration at 135 kg N ha⁻¹ in TIARA and no response in HAT. Possibly the activity of methylthioalkylmalate synthase 1 (MAM) and MAM3, which catalyzes condensations in the chain elongation process of homo-methionine and dihomo-methionine derived aliphatic GLSs (Sønderby et al. 2010b), responds differently to N and S, dependent on the cultivar. A S dependent response in MAM activity due to more substrate in the chain elongation process has previously been suggested (Fallovo et al. 2011), whereas our results propose genetically determined diverse N sensitivity. N effects on the indole glucobrassicin differed between all cultivars with decreasing and increasing concentrations in HAT and TIARA, respectively, and no response in Reflex. Furthermore, a trend of more glucobrassicin hydroxylation and methoxylation with increasing N application in TIARA is suggested. Higher susceptibility of indole GLSs to environmental influences (Brown et al. 2003) supports the diverse responses of indole GLSs among cultivars in contrast to uniform and possibly genetically determined responses to N of aliphatic GLSs within cultivar.
In a study of 148 kale landraces (*acephala* group), five clusters based on morphologic and agronomic traits differed in total GLS concentration and all individual GLSs except sinigrin (Cartea et al. 2008). Calculated from the agronomic traits described, the cluster with the highest edible yield contained the highest total GLS concentration, opposite TIARA in our

Figure 2. Effects of nitrogen (N) available (kg ha⁻¹) on individual glucosinolate concentrations (µg g⁻¹ DM) in the edible plant material of curly kale cultivars HAT, Reflex, and Tiara. GLI: glucoiberin, PRO: progoitrin, GLR: glucoraphanin, SIN: sinigrin, GLN: gluconapin, HY: 4-hydroxy glucobrassicin, GLB: glucobrassicin, ME: 4-methoxy glucobrassicin, NGLB: neoglucobrassicin, GST: gluconasturtiin. Different letters indicate significant differences ($p \leq 0.05$) in a general linear mixed model among N level means (n = 3) within glucosinolate. Bars are standard error.
study. The cluster constituted by the tallest plants and lower edible yields had lowest total GLS concentration but highest concentration of glucoiberin. The results suggested a similar relationship between agronomic traits and GLS content, but the growth patterns and GLS concentrations were not comparable. Stalk S status might be important to GLS concentration; internal sulfate pools in Arabidopsis have been documented mobile under S deficiencies due to high vacuolar transporter expression (Kataoka et al. 2004). However, the three cultivars showed no visible signs of S deficiency i.e. uniform chlorosis, and stalk S concentration level was positively related to leaf GLS concentration level rather than exhibiting an inverse relationship. These results indicated the necessity to examine growth pattern and biomass allocation of traditional and F1 hybrid curly kale cultivars to assess altered GLS composition in future research.

**Experiment II: N and S level effects.** The GLSs identified were similar to those resolved in Experiment I. S application increased the total GLS concentration substantially besides the percentage of the aliphatic GLS group within total GLS concentration, and decreased indole GLS percentage correspondingly (Figure 3B, $p = 0.00911$; Table 2).
Our results demonstrated responses in aliphatic and indole GLS percentages with increasing N levels, identical to Experiment I (Figures 1A and 3A). Similarly, Stavridou et al. (2012) and Rosen et al. (2005) showed a comparable response to S fertilization in field studies of broccoli and cabbage, but diverse effects were observed from N supplies. In another broccoli field trial, additional S supply did not increase GLS concentration due to sufficient base soil contents from the study onset (Jones et al. 2007). In the current study, an interaction between N and S levels was only found for gluconasturtiin (Table 2). Earlier investigations in other Brassica species grown in pots have demonstrated higher-level interactions between N and S applications in relationship to GLS concentration (Groenbaek and Kristensen 2014, Schonhof et al. 2007). Biogeochemical conditions are less constricted in the field, including N and S from mineralization of soil organic matter. Our results obtained under field conditions showed relatively higher total N uptake at low N fertilizer levels, and total S uptake in experiment II (40 kg S ha⁻¹ on average when no S was applied; data not included), which reflects influence from other soil properties under
field conditions contra e.g. pots or hydroponics. These observations might explain the reduced number of interactions between N and S as the N and S soil pool is influenced by other factors, e.g. organic matter content, in addition to fertilizers applied in field studies (Rosen et al. 2005). This emphasized the challenges in transfer of management methods to manipulate GLS biosynthesis from highly controlled environments to large-scale production under field conditions. Closely related N and S assimilation in plants often causes an interaction between N and S fertilizers in relationship to plant N and S concentrations, however, this interaction was not observed in the present study. APS reductase regulates the balance between S partitioning in primary or secondary metabolism, and controls 90% of the primary sulfate reduction, which leads to cysteine and glutathione, which both donate S in core GLS biosynthesis (Sønderby et al. 2010b, Vauclare et al. 2002). However, Mugford et al. (2011) reported that even though APS reductase capacity was reduced and resulted in sulfate accumulation, the GLS pathway end-products showed no decrease in a mutant arabidopsis. Therefore, although a close relationship existed between N and S assimilation, and primary and secondary S metabolism with respect to GLS biosynthesis; enzyme and substrate levels were under more complex regulation (Mugford et al. 2011), which was consolidated by multifaceted soil properties in the present study.
Table 2. Effects of nitrogen (N) and sulfur (S) available (kg ha\(^{-1}\)) on glucosinolate concentrations (µg g\(^{-1}\) DM) in the edible plant material of curly kale cultivar Reflex.

| N    | S    | GLI\(^a\) | SIN | GLR | GLN | PRO | GLB | HY | ME | NGLB | GST |
|------|------|-----------|-----|-----|-----|-----|-----|-----|----|-----|------|-----|
| 90   | 4    | 1041±290\(^a\) | 350±120 | 122±39 | 10±4 | 12±4 | 150±62 | 62±11 | 147±47 | 51±26 | 7±2 cd\(^c\) |
| 30   | 2214±176 | 860±204 | 279±37 | 40±8 | 30±8 | 260±61 | 112±24 | 199±4 | 59±6 | 20±5 a |
| 135  | 4    | 519±146 | 229±95 | 53±12 | 5±2 | 5±2 | 119±65 | 45±8 | 136±51 | 38±7 | 3±2 d |
| 30   | 1384±227 | 534±17 | 171±35 | 21±4 | 19±5 | 264±5 | 72±21 | 160±2 | 68±24 | 12±1 b |
| 185  | 4    | 1094±204 | 406±10 | 125±39 | 11±3 | 15±8 | 246±64 | 62±7 | 129±28 | 48±9 | 8±1 bcd |
| 30   | 1912±467 | 637±95 | 260±74 | 27±9 | 30±2 | 448±79 | 83±29 | 171±20 | 61±6 | 11±1 bc |

Significance

| N    | S    | GLI\(^a\) | SIN | GLR | GLN | PRO | GLB | HY | ME | NGLB | GST |
|------|------|-----------|-----|-----|-----|-----|-----|-----|----|-----|------|-----|
| N**d | S*** | ***      | *** | *** | *** | *** | **  | **  | NS | NS  | NS   | **  |
| N*S  | NS   | NS       | NS  | NS  | NS  | NS  | NS  | NS  | NS | NS  | NS   | *   |

\(^a\)For abbreviations please consult Figure 2. \(^b\)± standard deviation. \(^c\)Different letters indicate significant differences (\(p \leq 0.05\)) between S and N level means (\(n = 3\)) in a general linear model. \(^d\)NS: not significant; \(^*: p \leq 0.05\); \(^**: p < 0.01\); \(^***: p < 0.001\).
Studies on arabidopsis MYB transcription factors characterized the gene regulation of GLS biosynthesis, supporting the complex regulatory processes. No clear relationship between gene transcript levels and GLS accumulation in planta were found (Sønderby et al. 2010a). Despite the complex regulations of GLS biosynthesis, several studies have shown a S-dose dependent GLS response, and a linear relationship between N/S ratio and GLS concentration (Groenbaek and Kristensen 2014, Omirou et al. 2009, Schonhof et al. 2007, Stavridou et al. 2012).

**Growth patterns, flavonoid glycosides, and hydroxycinnamic acid derivatives**

**Experiment I: N level and cultivar effects.** Schmidt et al. (2010b) and Neugart et al. (2012) identified 71 flavonoid glycosides, and three main hydroxycinnamic acid derivatives which were also detected in HAT, Reflex, and TIARA. We selected 19 structurally different flavonoid glycosides (Table 3), including the main flavonol glycosides and related structures in kale, and the three main hydroxycinnamic acid derivatives to examine cultivar and N level effects on these phytochemicals.

Table 3. Identified flavonoid glycosides.

<table>
<thead>
<tr>
<th>Non-acylated monoglycoside</th>
<th>Isorhamnetin-3-O-D-glucoside</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-acylated diglycosides</td>
<td>Kaempferol-3-O-sophoroside</td>
</tr>
<tr>
<td></td>
<td>Isorhamnetin-3-O-D-glucoside-7-O-D-glucoside</td>
</tr>
<tr>
<td>Non-acylated triglycosides</td>
<td>Quercetin-3-O-sophoroside-7-O-D-glucoside</td>
</tr>
<tr>
<td></td>
<td>Kaempferol-3-O-sophoroside-7-O-D-glucoside</td>
</tr>
<tr>
<td></td>
<td>Isorhamnetin-3-O-sophoroside-7-O-D-glucoside</td>
</tr>
<tr>
<td></td>
<td>Isorhamnetin-3-O-triglucoside</td>
</tr>
<tr>
<td>Monoacylated diglycosides</td>
<td>Kaempferol-3-O-sinapoyl-sophoroside</td>
</tr>
<tr>
<td></td>
<td>Kaempferol-3-O-feruloyl-sophoroside</td>
</tr>
<tr>
<td>Monoacylated triglycosides</td>
<td>Kaempferol-3-O-hydroxyferuoyl-sophoroside-7-O-D-glucoside</td>
</tr>
<tr>
<td></td>
<td>Quercetin-3-O-sinapoyl-sophoroside-7-O-D-glucoside</td>
</tr>
<tr>
<td></td>
<td>Kaempferol-3-O-sinapoyl-sophoroside-7-O-D-glucoside</td>
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<tr>
<td></td>
<td>Kaempferol-3-O-feruloyl-sophoroside-7-O-D-glucoside</td>
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<tr>
<td></td>
<td>Kaempferol-3-O-coumaroyl-sophoroside-7-O-D-glucoside</td>
</tr>
<tr>
<td>Monoacylated tetracygosides</td>
<td>Kaempferol-3-O-sinapoyl-sophoroside-7-O-diglucoside</td>
</tr>
<tr>
<td></td>
<td>Kaempferol-3-O-feruloyl-sophoroside-7-O-diglucoside</td>
</tr>
<tr>
<td></td>
<td>Quercetin-3-O-sophoroside-7-O-sinapoyl-diglucoside</td>
</tr>
<tr>
<td>Diacylated tetracygosides</td>
<td>Quercetin-3-O-disinapoyl-triglucoside-7-O-D-glucoside</td>
</tr>
<tr>
<td></td>
<td>Kaempferol-3-O-disinapoyl-triglucoside-7-O-D-glucoside</td>
</tr>
</tbody>
</table>
Kaempferol glycosides were the main constituent in the three kale cultivars, followed by hydroxycinnamic acid derivatives, and quercetin glycosides (Table 4). Quercetin and total flavonoid glycosides decreased with increasing N application in Reflex. This was in line with the results of Stewart et al. (2001) who found decreasing concentrations of quercetin and total flavonoid aglycones as well as kaempferol and isorhamnetin in arabidopsis when increasing N levels were applied. Furthermore, another arabidopsis study revealed a broad and clear coordinated response to NO₃⁻ addition in genes controlling phenylpropanoid and flavonoid metabolism, as well as primary metabolism, suggesting a shift away from C-rich phytochemicals, such as flavonols (Scheible et al. 2004). This corresponded to the balance between N and C in relationship to primary and secondary metabolism as discussed in connection to GLSs.

Table 4. Effects of nitrogen (N) available (kg ha⁻¹) on flavonoid glycoside and total hydroxycinnamic acid derivative concentrations (mg g⁻¹DM) in the edible plant material of curly kale cultivars HAT, Reflex, and TIARA.

<table>
<thead>
<tr>
<th>N</th>
<th>CV</th>
<th>Kaempferol glycosides</th>
<th>Quercetin glycosides</th>
<th>Isorhamnetin glycosides</th>
<th>Total flavonoid glycosides</th>
<th>Total hydroxycinnamic acid derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>HAT</td>
<td>6.43±0.69</td>
<td>1.89±0.58</td>
<td>1.05±0.69</td>
<td>9.37±0.60</td>
<td>2.39±0.09</td>
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<tr>
<td>230</td>
<td></td>
<td>5.60±0.96</td>
<td>1.37±0.23</td>
<td>0.62±0.11</td>
<td>7.58±0.97</td>
<td>2.20±0.38</td>
</tr>
<tr>
<td></td>
<td>N effect</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>90</td>
<td>Reflex</td>
<td>5.39±0.08</td>
<td>3.52±0.34 a</td>
<td>0.20±0.02</td>
<td>9.12±0.44 a</td>
<td>2.15±0.02</td>
</tr>
<tr>
<td>135</td>
<td></td>
<td>4.98±0.28</td>
<td>2.99±0.20 ab</td>
<td>0.19±0.01</td>
<td>8.16±0.33 b</td>
<td>2.12±0.17</td>
</tr>
<tr>
<td>185</td>
<td></td>
<td>5.32±0.18</td>
<td>2.74±0.34 b</td>
<td>0.21±0</td>
<td>8.27±0.49 ab</td>
<td>2.08±0.15</td>
</tr>
<tr>
<td>230</td>
<td></td>
<td>5.22±0.35</td>
<td>2.36±0.26 b</td>
<td>0.21±0.02</td>
<td>7.79±0.57 b</td>
<td>1.90±0.11</td>
</tr>
<tr>
<td></td>
<td>N effect</td>
<td>NS</td>
<td>**</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>90</td>
<td>TIARA</td>
<td>6.71±2.34</td>
<td>2.87±0.76</td>
<td>0.67±0.14</td>
<td>10.26±2.39</td>
<td>3.51±1.08</td>
</tr>
<tr>
<td>135</td>
<td></td>
<td>6.87±0.72</td>
<td>3.21±1.55</td>
<td>0.64±0.08</td>
<td>10.72±2.34</td>
<td>3.48±0.52</td>
</tr>
<tr>
<td>185</td>
<td></td>
<td>6.47±1.39</td>
<td>1.68±0.57</td>
<td>0.82±0.21</td>
<td>8.97±2.03</td>
<td>4.09±1.05</td>
</tr>
<tr>
<td>230</td>
<td></td>
<td>5.43±1.20</td>
<td>1.67±0.55</td>
<td>0.60±0.18</td>
<td>7.70±1.83</td>
<td>3.52±0.12</td>
</tr>
</tbody>
</table>

*a± standard deviation. *: p ≤ 0.05; **: p < 0.01 in a general linear model among N level means (n=3) within chemical group.
Higher quercetin glycoside and lower isorhamnetin glycoside concentrations in Reflex, as compared to HAT ($p < 0.0001$ and $p < 0.01$, respectively) indicated decreased quercetin to isorhamnetin conversion in Reflex. Possibly due to reduced activity of the responsible enzyme, quercetin-3-O-methyltransferase, which is suggested not to be affected by N concentration (Fallovo et al. 2011). Cultivar differences in our study do not support this as both Reflex and HAT showed lower leaf N concentration and Reflex seemed to be more responsive to N fertilization with respect to phytochemical modulation as well as biomass production. A study on two F1 kale hybrids revealed no changes in phenolic compound concentrations when application of N increased although yield increased in one of the cultivars (Lata 2014). Kaempferol is a precursor to quercetin, and isorhamnetin is a methylated product of quercetin, therefore the lack of response of kaempferol and isorhamnetin to increased N levels might be due to the absence of a catechol structure in the B-ring, and consequently a lower antioxidant activity (Fiol et al. 2012). However, both increases and decreases in concentrations of individual kaempferol flavonoids were revealed in tronchuda cabbage ($B. oleracea$ L. var. $costata$ DC.) grown under moderate and high N levels (Sousa et al. 2008). Variations were due to harvest time and plant part, with no consistency, suggesting additional ontogenetic and environmental influences. Abovementioned indicates that the F1 hybrid Reflex was more responsive to N fertilization compared to TIARA and HAT. This could be explained by breeding focusing on increased biomass production, which strictly relate to N responsiveness and possibly corresponds to the clear response to N fertilization as the growth patterns revealed in the present study.

N level had no effect on total hydroxycinnamic acid derivatives (Table 4). TIARA had the highest concentration of hydroxycinnamic acid derivatives, as well as isorhamnetin glycosides compared to Reflex ($p < 0.0001$). This might reflect different cultivar yields, like for the GLSs. However, cinnamic acid is the flavonoid and hydroxycinnamic acid precursor, and its biosynthesis is controlled by phenylalanine ammonia lyase (PAL) activity, which is responsible for phenylalanine conversion into cinnamic acid in flavonol biosynthesis (Hahlbrock and Grisebach 1979). Increased N levels have been found to reduce PAL activity (Ibrahim et al. 2011). Therefore elevated leaf N concentration in TIARA does not support our results. Furthermore, high N concentration and reduced plant growth might shift secondary metabolism towards N-containing phytochemicals, as argued earlier. Simple linear regression results between flavonoid glycoside groups or total hydroxycinnamic acid derivatives, and either leaf N concentration or total N uptake revealed no relationships between these parameters for any of the cultivars (data not included). Inconsistent with our results, Fallovo et al. (2011) found a negative linear
relationship between quercetin, kaempferol as well as total flavonoids, and N concentration in *Brassica rapa* and *Brassica juncea* leaves. However, in Reflex a negative linear relationship between edible yield and kaempferol, quercetin, and total flavonoid glycosides was detected ($p = 0.0345$, $R^2 = 0.18$; $p < 0.0001$, $R^2 = 0.52$ and $p < 0.0001$, $R^2 = 0.58$, respectively). These results support the effects of N application and growth pattern on flavonoid glycosides and hydroxycinnamic acid derivatives; if we assume Reflex was N but not C limited at high edible yield levels.

Distinct growth patterns among the three kale cultivars were associated to a diverse non-, mono-, and di-acylated, mono-, di- or tetracylglycosylated flavonoid composition ($p < 0.001$). While the non-acylated monoglycoside (isorhamnetin-3-O-D-glucoside) was only detected in HAT, the non-acylated diglycosides were comparable in the traditional cultivars HAT and TIARA. Lower concentrations were resolved in the F1 hybrid Reflex ($p < 0.001$), possibly due to higher edible yield. Main kale flavonoid glycoside the monoacylated tri- and tetracylglycosides decreased in concentration with increasing N application in Reflex (Figure 4). Likewise the minor monoacylated diglycosides decreased in TIARA. Schmidt et al. (2010a) reported similar cultivar effects on flavonoid glycosides in kale as a response to changing temperature and radiation. The different responses to N application in leaf N concentrations of Reflex and TIARA might therefore explain the variable effects on monoacylated diglycosides and monoacylated tri- and tetracylglycosides. A study in cauliflower showed a higher kaempferol-3-diglucoside-7-diglucoside concentration with mineral fertilizer application containing less total N due to a high share of compost compared to the other cultivation methods (Martinez-Blanco et al. 2011). Diacylated tetracylglycoside concentrations in TIARA seemed to follow elevated leaf N level patterns at 135 and 230 kg N ha$^{-1}$ applications. This was inconsistent with the results of Scheible et al. (2004) suggesting less C-rich phytochemicals under high NO$_3^-$ levels.
We propose a different response to excess N uptake in TIARA, since glucose molecules, as a result of primary metabolism, possibly lead to glycosylation resulting in higher diacylated tetracyglycoside concentrations. Fritz et al. (2006) found elevated glucose concentration in
N-replete compared to N-deficient tobacco plants (*Nicotiana tabacum* L.), supporting our suggestion. Results did not reveal differences in free glucose levels among cultivars and N levels (0.82 mg g⁻¹ DM on average, data not included), which indicates all cultivars had the same threshold for free glucose, in contrast to the moiety levels in GLS or flavonoid glycoside molecules, which differed between cultivars. In summary, monoacylated diglycosides and monoacylated tri- and tetragnosides decreased with increasing N application in TIARA and Reflex, respectively. The main kale glycosides, i.e. non-acylated triglycosides were not affected.

In conclusion, genetic differences between traditional and F1 hybrid cultivars were reflected in cultivar growth patterns and N responses, and could be associated to phytochemical composition and concentration. Our results emphasize that N and S fertilizer management and cultivar selection can be used as a tool for optimization of GLS and flavonoid concentrations as well as edible yield. However, the target of interest subsequently defines the choice of cultivar and fertilizer strategy. Furthermore, it points to the positive perspective of re-introduction of traditional kale cultivars with health beneficial compounds, and additionally the potential role of phytochemicals related to sensory properties, as reported by Jensen et al. (2014).

**Abbreviations used**

APS, adenosine 5’-phosphosulfate; C, carbon; DM, dry matter; GLB, glucobrassicin; GLI, glucoiberin; GLN, gluconapin; GLR, glucoraphanin; GLS, glucosinolate; GST, gluconasturtiin; HAT, Høj Amager Toftø; HY, 4-hydroxy glucobrassicin; LS, least squares; MAM, methylthioalkylmalate synthase; ME, 4-methoxy glucobrassicin; MeOH, methanol; MYB, myeloblastosis; N, nitrogen; NGLB, neoglucobrassicin; PAL, phenylalanine ammonia lyase; PRO, progoitrin; S, sulfur; SIN, sinigrin; TIARA, Halvhøj Ekstra Moskruset Tiara.

**Acknowledgment**

We are grateful to Astrid Bergmann, Jens Barfod, and Knud Erik Pedersen for performing the field experiments, and to Birgitte Foged and Antje Bamberg for contributing to the chemical analyses.
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This work was a part of the “MaxVeg” project financed by The Danish Council for Strategic Research´s Programme Commission on Health, Food and Welfare.
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Chapter 5

5.1 Paper IV

Curly kale (*Brassica oleracea* L. var. *sabellica* L.) cultivar selection grown under different nitrogen and sulfur supplies in the field. II. Variations in the content of phytochemicals and how it is reflected in the sensory properties

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Has been rewritten and resubmitted after an invitation to Journal of Agricultural and Food Chemistry. Submitted as a joint manuscript with paper III
Abstract

The overall aim of this study was to investigate whether the content of health beneficial glucosinolates (GLSs) and total phenolics could be increased in curly kale without changing the sensory properties of sweetness, bitterness, mouthfeel and texture. Analysis of simple sugars was included because sugar is known to affect perceived sweetness and bitterness. Different strategies (cultivar selection including F1 hybrid and traditional cultivars (analysis 1), different fertilization approaches (analysis 2) and a combination of the two (analysis 3 and 4)) were employed and succeeded in producing curly kale with large variations in GLS, sugar and total phenolic contents. Sweetness was well predicted by sugar content, while GLS and total phenolic contents could not consistently be correlated to any of the sensory properties. Consequently, there is potential for producing kale with high GLS and phenolic contents without changing the sensory properties.

Keywords

Brassicaceae; glucosinolates; total phenolics; sensory properties; simple sugars
Introduction

Curly kale (*Brassica oleracea* L. var. *sabellica* L.) is a vegetable with a high nutritional value. Kale is included in a range of Danish traditional dishes. It is experiencing a revival as a consequence of the increased focus on ‘New Nordic Food’ where one of the aims is: ‘to base our cooking on ingredients and produce whose characteristics are particularly in our climates’ (New Nordic Food 2014). In New Nordic Food, there is focus on the sensory properties as well as on the health benefits associated with a high intake of vegetables.

Curly kale belongs to the plant family, *Brassicaceae*. This family contains more than 350 genera and 3000 species (Fahey et al. 2001) and all the investigated species are found to be able to synthesize glucosinolates (GLSs) (Kjaer 1976). The individual GLSs can, based on their precursor amino acid and the types of modifications to the R-group, be classified as either aliphatic, aromatic or indole GLSs (Halkier and Gershenzon 2006). Previously identified GLSs in kale are the aliphatic GLSs: glucoraphanin (4-methylsulphinylbutyl GLS), glucoiberin (3-methylsulphinylpropyl GLS), sinigrin (2-propenyl GLS), progoitrin ((R)-2-hydroxy-3-butenyl GLS), epiprogoitrin ((S)-2-hydroxy-3-butenyl GLS), and gluconapin (3-butenyl GLS), the aromatic GLS: gluconasturtiin (2-phenylethyl GLS) and the indole GLSs: glucobrassicin (3-indolymethyl GLS), neoglucobrassicin (1-methoxy-3-indolymethyl GLS), 4-hydroxy glucobrassicin (4-hydroxy-3-indolymethyl GLS) and 4-methoxy glucobrassicin (4-methoxy-3-indolymethyl GLS) (Kushad et al. 2004, Velasco et al. 2007, Velasco et al. 2011). Upon cellular disruption, the thioglucoside linkages of GLSs are hydrolysed to various bioactive breakdown products by the endogenous enzyme myrosinase (Fahey et al. 2001, Halkier and Gershenzon 2006, Kjaer 1976).

Over the past couple of decades, compelling evidence has linked increased consumption of vegetables, especially *Brassica* vegetables, to a lowered prevalence of several types of cancer (Block et al. 1992, Traka and Mithen 2009, Verhoeven et al. 1997, Verkerk et al. 2009). The lowered risk of cancer linked to the intake of *Brassica* vegetables is positively associated with the content of GLSs (Verhoeven et al. 1997). The GLS content varies both quantitatively and qualitatively between varieties and cultivars. Schonhof and co-workers (2004) showed that both the individual and total content of GLSs vary greatly among different species of the *Brassica* genus. Changes in the fertilization approach are another powerful method to manipulate the GLS content. In field trials, sulfur (S) and nitrogen (N) fertilization and the ratio between them have previously been shown to influence the GLS content in *Brassica* vegetables (Jones et al. 2007, Rosen et al. 2005, Stavridou et al. 2012). Phenolics are another important group of phytochemicals present in kale. The health
benefits associated with phenolics is generally recognized to be because of their high antioxidative activity (Crozier et al. 2009, Yao et al. 2004). A number of factors influence the content of phenolics in plants, such as cultivar, fertilization and stage of plant development (Biesiada et al. 2010, Korus 2011).

Many of the GLSs, as well as their degradation products and phenolics, are known to be bitter and pungent – sensory properties that are negatively correlated with consumer preferences (Drewnowski and Gomez-Carneros 2000, van Doorn et al. 1998). This relationship between high GLS and phenolics contents and the adverse effect on sensory properties poses a challenge in the effort to optimize the health potential of kale. However, the perceived bitterness and pungency of GLSs and phenolics are influenced by many other constituents naturally present in kale, with sugar as one of the most important ones (Beck et al. 2014, Keast et al. 2004, Ley 2008, Zabaras et al. 2013). Sugar is widely recognized to mask bitterness (Keast 2008, Keast et al. 2004, Lawless 1979, Ley 2008) and sweetness is generally known to be a positive driver for vegetable liking (Cox et al. 2012, Dinehart et al. 2006, Schonhof et al. 2004). Moreover, sweetness is found to be an important contributor to the taste of cabbage (Cox et al. 2012, Zabaras et al. 2013). This is also expected to be true for kale, which has a relatively high natural sugar content of 2.06 g per 100 g fresh weight (FW) (Danish Food Composition Databank 2014). Similar to GLS and total phenolic contents, sugar content depends on cultivar and can be manipulated by using different fertilization approaches (Kano et al. 2007, Schonhof et al. 2004).

A better understanding of how specific phytochemicals or groups of phytochemicals influence the sensory properties in the actual vegetable matrix is crucial in order to produce vegetables of the highest nutritional and sensory quality. Such a study relies on the ability to produce vegetables that differ in the content of phytochemicals. The link between phytochemical content and the sensory quality of kale has to these authors’ knowledge never been investigated before.

The overall aim of this study was to investigate whether changes in phytochemical content in the form of GLSs and total phenolics in curly kale were directly linked to changes in sensory properties. Based on three field experiments, curly kale with large variations in phytochemical contents and sensory properties was produced by employing different strategies: cultivar selection (analysis 1), changes in the fertilization approach (analysis 2) and a combination of these two strategies (analysis 3 and 4). To fulfil the aim, a two-step procedure was applied. The first step was to identify the strategy providing the largest deviation in sample material. Focus was on the range obtained in the content of individual
phytochemicals and in sensory intensities of curly kale from the four analyses. The second step involved a multivariate approach to study the influence of individual phytochemicals or groups of phytochemicals on the sensory properties of curly kale.

**Materials and methods**

**Chemicals.** Glucoiberin, progoitrin, glucoraphanin, gluconapin, sinigrin, hydroxyglucobrassicin, glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin were obtained from C2 Bioengineering ApS (Karlslunde, Denmark); sinigrin and goitrin from Alfa Aesar (Massachusetts, US); glucotropaeolin from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany); acetic acid, barium acetate, calcium glycerophosphate hydrate, methanol, sulfatase and sodium hydroxide, fructose and sucrose were obtained from Sigma-Aldrich (Steinheim, Germany) and glucose was obtained from Merck Chemicals (Darmstadt, Germany).

**Experimental design.** The study encompassed examination of phytochemicals and sensory properties of kale from four analyses based on three field experiments. Evaluation of the phytochemical content was centred on the content of GLSs and total phenolics. Analyses of simple sugars and dry matter content (DM) was also included because sugar is known to influence the sensory properties. Phytochemicals and the sensory properties were studied for different cultivars of kale (analysis 1 and 3) and kale grown under different fertilization approaches (analysis 2, 3 and 4). The cultivars included in this study were based on the selection provided by NordGen germplasm collection (NordGen 2014) together with the modern F1 hybrid ‘Reflex’, while the choice of fertilization approach was based on existing literature and pre-trials in the field. The field experiments were set out in a completely randomized block design with three replications of plots grown with kale on a sandy loam (Typic Agrudalf) at Department of Food Science in Aarslev, Denmark (55°18'N, 10°27'E). From each plot three representative plants were harvested within an appropriate guard area and the samples were kept in closed plastic bags in a cold store at 1 °C for a maximum of four days before further analyses. Additional details of the field experiments can be found in Groenbaek et al. (2014) (Paper III).
Plant material

**Analysis 1:** In 2010, eight cultivars of kale were planted in the field on June 18\(^{th}\) and harvested at maturity on November 2\(^{th}\). The eight cultivars were a modern F1 hybrid, ‘Reflex’, and seven traditional Danish cultivars from the NordGen germplasm collection: ‘Lav Opretvoksende’ (LAVO) accession 545, ‘Halvhøj Kruset Bona’ (BONA) accession 1831, ‘Halvhøj Kruset Konserva’ (HKK) accession 1832, ‘Halvhøj Ekstra Moskruset Tiara’ (TIARA) accession 1833, ‘Lav Kruset Harvester’ (LKH) accession 1834, ‘Høj Amager Sundby Torve’ (HAST) accession 1836 and ‘Høj Amager Toftø’ (HAT) accession 4085. The cultivars were grown according to standard practice with 135 kg N ha\(^{-1}\) and no S.

**Analysis 2:** In 2011, the Reflex cultivar was grown under different fertilization conditions, receiving two different S levels (4 or 30 kg ha\(^{-1}\)) and three N levels (90, 135 or 185 kg ha\(^{-1}\)). The kale was planted on May 5\(^{th}\) and harvested at maturity on October 10\(^{th}\).

**Analysis 3:** In 2011, three cultivars of kale (Reflex, HAT and TIARA) were grown in soil fertilized with two different N levels (90 and 230 kg ha\(^{-1}\)) and an S level of 30 kg ha\(^{-1}\). The kale was planted on May 3\(^{rd}\) and harvested at maturity on October 10\(^{th}\).

**Analysis 4:** In 2011, two cultivars of kale (Reflex and TIARA) were grown in soil fertilized with four different N levels (90, 135, 185 and 230 kg ha\(^{-1}\)) and an S level of 30 kg ha\(^{-1}\). The kale was planted on May 3\(^{rd}\) and harvested at maturity on October 10\(^{th}\).

Analysis 3 and 4 were based on a common experimental setup in the field, but sampling and sensory evaluations were done independently. Analysis 2 and analysis 3 and 4 correspond to Experiment II and I in the work by Groenbaek and co-authors (2014) (Paper III).

**Sample preparation.** Kale leaves from the second, fourth and sixth rosette of leaves (top down) were separated from the stalk by snapping off the stems at a diameter of approximately 7 mm. The kale leaves were rinsed thoroughly in cold tap water and excessive water shaken off. The kale leaves were finely chopped into approximately 1 x 0.2 x 0.2 cm pieces using a bowl cutter model BS from Talleres Ramón, S. L. (Barcelona, Spain) two-three hours prior to the sensory profiling. For the chemical analyses, samples were frozen in liquid N immediately after chopping and subsequently kept at -24 °C until freeze-drying (three-four days under vacuum) using a CHRIST freeze-dryer, Gamma 1-20 (Osterode am Harz, Germany). Prior to chemical analysis, samples were ground to an
ultimate fineness of 0.5 mm using an Ultra Centrifugal mill ZM 200 from Retsch (Haan, Germany).

**Chemical measurements**

**Determination of glucosinolates.** The GLS content was determined in accordance with the method described in Beck et al. (2014).

**Analysis of total phenolics.** Total phenolics was analyzed using the rapid Folin Ciocalteu (FC) method introduced by Magalhães and co-worker (2010) following the approach described by Bach et al. (2013). Results are presented as gallic acid equivalents (GA).

**Determination of simple sugars.** Free sugars (sucrose, fructose and glucose) were analyzed following the same procedure as Beck et al. (2014) with one small modification: in the present study 0.25 mL of the sample extract, and not 0.50 mL, was diluted with distilled water to a total volume of 25.0 mL. Sugars were quantified by use of calibration curves of commercial authentic standards.

**Dry matter content.** The dry matter (DM) determination was based on a gravimetrical procedure. Samples were scaled before and after drying (80 °C, 18 h) in a ventilated oven from Lytzen A/S (Herlev, Denmark).

**Sensory profiling.** Sample material, in amounts of 12 g, was served in transparent plastic trays from ABENA A/S (Aabenraa, Denmark) with matching plastic lids labelled with a unique, random three-digit number. The panel consisted of 9-11 assessors (7-9 females/2 males, aged from 26 to 61). The sensory profiling took place in a laboratory complying with the requirements of the ASTM International (ASTM, 1986) with a sensory panel that had been tested and trained according to international standard ISO 8586-1 (ISO, 1993). In the initial phase, assessors were presented with reference samples for the different characteristics relevant for the sensory properties of kale, together with a sub-set of the kale samples included in the profile. From these samples, assessors generated a list of attributes to be used in the sensory profiling. Attributes, together with a definition of each attribute, are provided in Table 1. Assessors participated in 3-6 two-hour training sessions before the actual test. Feedback on individual and panel performance was given after each training session with the aim of improving and standardizing the discriminating power of the panel. Feedback was based on calculations and plots made in PanelCheck v. 1.4.0 (www.PanelCheck.com).
Table 1. List of attributes and definition of attributes

<table>
<thead>
<tr>
<th>Group</th>
<th>Descriptor</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taste</td>
<td>Sweetness</td>
<td>The basic sweet taste. Sweetness is to be evaluated after chewing the sample 4 times</td>
</tr>
<tr>
<td></td>
<td>Bitterness</td>
<td>The basic bitter taste. The taste is most dominate after the sample has been chewed well</td>
</tr>
<tr>
<td>Sensation</td>
<td>Pungency (nose)</td>
<td>A prickly sharp sensation in the tissue in the nose similar to the one experienced when evaluating the aroma of cress</td>
</tr>
<tr>
<td></td>
<td>Pungency (mouth)</td>
<td>A prickly sharp sensation in the tissue in the nose similar to the one experienced when chewing cress</td>
</tr>
<tr>
<td></td>
<td>Astringency</td>
<td>Drying-out, roughening, and puckery sensation felt in the mouth</td>
</tr>
<tr>
<td>Texture</td>
<td>Juiciness</td>
<td>The amount of juice released on mastication</td>
</tr>
<tr>
<td></td>
<td>Crispiness</td>
<td>The degree of which the sample is fractured. Clean and total fracture.</td>
</tr>
</tbody>
</table>

Samples were analyzed in triplicate in three blocks, with one repetition for each block. Within each block, samples were served randomly in order to avoid bias. The assessors evaluated samples at an individual rate on a 15.0-cm line scale with endpoint anchors labelled ‘low’ on the left and ‘high’ on the right. Ratings were registered directly into a registration system from Fizz software, 2.30C, Biosystemes (Couternon, France).

**Statistical analysis.** Statistical analyses were performed using the freeware programs PanelCheck v. 1.4.0 (www.PanelCheck.com) and R (ver. 2.13.1, R Development Core Team) together with SIMCA-P+ Ver. 12 from Umetrics (Umeå, Sweden). PanelCheck was used for assessor evaluations and for a mixed three-way analysis of variance (ANOVA) of the sensory data, while one-way ANOVA was used to analyse chemical data in R. Statistical significance was defined at $p \leq 0.05$. Multivariate analysis in the form of partial least squares (PLS) regression was performed in Simca-P+ on mean data in order to study the relationship between chemical (X-matrix) and sensory (Y-matrix) measurements in terms of prediction of Y-variables from X-variables. Data were auto-scaled and the models were performed with full cross-validation. The results from the PLS regression were interpreted by inspection of the Variable Importance in Projection (VIP) scores. The VIP scores estimate the importance of each variable in the projection used in a PLS regression model, in this case the relevance of the chemical compounds for the sensory properties. A variable with a VIP score close to or higher than 1 can be considered important in the given model (Chong and Jun 2005).
Results and discussion

Analysis 1 that included eight different cultivars of kale was conducted in 2010 while the analysis 2 on the differences in fertilization approach and analysis 3 and 4 on a combination of different cultivars and fertilization approaches were conducted in 2011. It is commonly acknowledged that parameters that are not uniform across harvest years, such as climate, water availability and light, affect the content of phytochemicals (Björkman et al. 2011). Some of the differences observed in analysis 1 and analysis 2-4 may therefore be due to climatic differences in harvest years. However, as the different strategies were mainly applied to obtain sample material with a large spread in the content of phytochemicals, this was not critical since no direct comparisons of the analyses from different harvest years will be attempted.

Chemical analyses

Glucosinolates. Analysis 1 had the largest spread in individual GLS contents with four out of the 10 identified GLSs having a wide spread compared to analysis 2-4 (Table 2). The four GLSs were: progoitrin, glucoraphanin, sinigrin and 4-methoxy glucobrassicin, which obtained a span in content (in mg 100 g⁻¹ FW) of 1.86, 42.63, 31.68 and 5.54, respectively. The largest span in total GLS content (63.32 mg 100 g⁻¹ FW) was also observed in analysis 1, although closely followed by analysis 2 (49.68 mg 100 g⁻¹ FW) and analysis 4 (48.53 mg 100 g⁻¹ FW). Analysis 2, where the effect of different levels of N and S supply was tested, exhibited the largest spread in the content of glucoiberin (28.76 mg 100 g⁻¹ FW) and 4-hydroxy glucobrassicin (1.14 mg 100 g⁻¹ FW), while the spread in glucobrassicin content (12.75 mg 100 g⁻¹ FW) and neoglucobrassicin (1.32 mg 100 g⁻¹ FW) was largest in analysis 4 (Table 2). The results confirm that all the employed strategies succeeded in producing kale differing greatly in GLS content and that the biosynthesis of the individual GLSs responded differently to different strategies.

Comparisons of results from analysis 2-4 showed that changes in both N and S supply is more effective in generating differences in GLS content in kale (Reflex) than only varying the N level. This is further discussed in the work performed by Groenbaek and co-workers (2014) (Paper III).
Table 2. Concentration range of the content of glucosinolates (mg 100 g⁻¹ fresh weight kale), sugars and dry matter (g 100 g⁻¹ fresh weight kale) and total phenolics (mg GA equivalents 100 g⁻¹ fresh weight kale) measured in kale from analysis 1-4. The spread in concentrations is only shown for compounds obtaining significant product effect (p ≤ 0.05) within each analysis.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Analysis 1</th>
<th>Analysis 2</th>
<th>Analysis 3</th>
<th>Analysis 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Field</td>
<td>Field</td>
<td>Field</td>
<td>Field</td>
</tr>
<tr>
<td></td>
<td>experiment 1</td>
<td>experiment 2</td>
<td>experiment 3</td>
<td>experiment 3</td>
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<tr>
<td></td>
<td>2010</td>
<td>2011</td>
<td>2011</td>
<td>2011</td>
</tr>
<tr>
<td>8 Cultivars</td>
<td>1 Cultivar</td>
<td>3 N Levels</td>
<td>2 Cultivars</td>
<td>2 Cultivars</td>
</tr>
<tr>
<td>2 N levels</td>
<td>3 N levels</td>
<td>2 N levels</td>
<td>N levels</td>
<td>N levels</td>
</tr>
<tr>
<td>Glucoiberin</td>
<td>8.8-37.6</td>
<td>5.0-19.8</td>
<td>0.1-1.8</td>
<td>0.3-2.8</td>
</tr>
<tr>
<td>Progoitrin</td>
<td>0.1-2.0</td>
<td>0.1-0.5</td>
<td>0.2-3.2</td>
<td>1.3-8.2</td>
</tr>
<tr>
<td>Glucoraphanin</td>
<td>0.4-43.1</td>
<td>0.9-4.7</td>
<td>0.9-4.7</td>
<td>4.9-27.0</td>
</tr>
<tr>
<td>Sinigrin</td>
<td>4.9-36.5</td>
<td>3.9-14.6</td>
<td>4.9-27.0</td>
<td>4.9-27.0</td>
</tr>
<tr>
<td>Gluconapin</td>
<td>0.1-0.7</td>
<td>0.1-0.7</td>
<td>0.1-0.7</td>
<td>0.1-0.7</td>
</tr>
<tr>
<td>4-Hydroxy glucobrassicin</td>
<td>0.6-1.4</td>
<td>0.8-1.9</td>
<td>0.3-2.8</td>
<td>2.3-5.1</td>
</tr>
<tr>
<td>Glucobrassicin</td>
<td>4.5-15.9</td>
<td>2.0-7.9</td>
<td>4.1-16.8</td>
<td>4.1-16.8</td>
</tr>
<tr>
<td>4-Methoxy glucobrassicin</td>
<td>3.9-9.4</td>
<td>2.0-4.4</td>
<td>2.3-5.1</td>
<td>2.3-5.1</td>
</tr>
<tr>
<td>Neoglucobrassicin</td>
<td>0.0-0.2</td>
<td>0.7-1.2</td>
<td>0.6-1.9</td>
<td>0.6-1.9</td>
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<tr>
<td>Gluconasturtii</td>
<td>NAa</td>
<td>0.0-0.3</td>
<td>0.0-0.3</td>
<td>0.0-0.3</td>
</tr>
<tr>
<td>Total glucosinol</td>
<td>30.1-93.4</td>
<td>24.2-73.9</td>
<td>31.4-79.9</td>
<td>31.4-79.9</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.1-1.4</td>
<td>1.1-1.4</td>
<td>1.1-1.4</td>
<td>1.1-1.4</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.9-1.2</td>
<td>0.9-1.2</td>
<td>0.9-1.2</td>
<td>0.9-1.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.4-0.9</td>
<td>0.4-0.9</td>
<td>0.4-0.9</td>
<td>0.4-0.9</td>
</tr>
<tr>
<td>Total sugar</td>
<td>2.4-3.3</td>
<td>2.4-3.3</td>
<td>2.4-3.3</td>
<td>2.4-3.3</td>
</tr>
<tr>
<td>Dry matter</td>
<td>14.5-17.6</td>
<td>14.7-17.5</td>
<td>14.7-17.5</td>
<td>14.7-17.5</td>
</tr>
<tr>
<td>Total phenolics</td>
<td>150.9-239.0</td>
<td>136.4-237.6</td>
<td>136.4-237.6</td>
<td>136.4-237.6</td>
</tr>
</tbody>
</table>

aNA: Not analyzed

**Total phenolics.** The FC assay used in this study is an unspecific measurement of phenolics as the FC reagent also reacts with other antioxidative compounds. In plants, phenolics are the most abundant group of antioxidants and the FC is thus recognized as an appropriate method for estimating the content of phenolics (Everette et al. 2010). The total content of phenolics ranged from 136-239 mg GA 100 g⁻¹ FW in the four analyses (data not shown), which is at the lower end of the scale compared to results obtained by Korus (2011) who measured between 256 and 531 mg chlorogenic acid equivalents 100 g⁻¹ FW kale (*Brassica oleracea* L. var. *acephala*), but is at the higher end compared to the level reported by Olsen and co-workers (2012). However, due to the use of different reference
compounds – gallic acid in the present study and chlorogenic acid in the two cited studies (Korus 2011, Olsen et al. 2012) – the results cannot be directly compared. The FC method clearly discriminated the total phenolics content of samples in analysis 1 and 4. The largest spread of 101.22 mg GA 100 g⁻¹ FW in total phenolics was obtained in analysis 4 compared to 88.10 mg GA 100 g⁻¹ FW for analysis 1 (Table 2). However, most of the variation in the polyphenol concentration in analysis 4 could be ascribed to an inter-cultivar difference, since the mean concentration achieved for Reflex was 151±10 mg GA 100 g⁻¹ FW compared to 213±16 mg GA 100 g⁻¹ FW for TIARA (data not shown). Biesiada and co-workers (2010) found that an increased N level contributed to a decrease in the phenolics content in red cabbage (Brassica oleracea L. var. capitata ‘Langendijker’ cultivar), whereas inter-cultivar differences were found to be the main contributor to differences in total phenolics content in kale in the present study. An effect of N level was plausible due to the known regulatory effect of N on phenylalanine ammonia lyase in the phenylpropanoid pathway leading to synthesis of phenolics (Ibrahim et al. 2011). However, the cultivar effect was larger, possibly caused by a large difference in the growth and biomass partitioning of the cultivars (Paper III).

**Sugars and dry matter.** Only in analysis 1 did the glucose, fructose, sucrose and total sugar content differ significantly within kale samples. The spread in concentration was 0.29 g 100 g⁻¹ FW for fructose, 0.33 for glucose, 0.53 for sucrose and 0.92 for the total content of simple sugars (Table 2). Obviously, fertilization with N and S at the levels applied in analysis 2-4 did not influence sugar content. The DM content followed the same tendency as for sugars, since significant product effects were only observed for the two experiments including three or more cultivars (analysis 1 and 3) (Table 2).

**Sensory profiling**

Results from the sensory profiling showed that the largest number of attributes to significantly differentiate kale cultivars was obtained in analysis 1 where all attributes except pungency (nose) had a significant effect (Table 3). In analysis 2, four attributes (bitterness, pungency (nose), pungency (mouth) and astringency) had a significant effect in differentiating the tested kale, whereas for analysis 3 and 4, respectively two attributes (pungency (nose) and juiciness) and one attribute (pungency (nose)) had a significant effect. In analysis 1, the range within which attributes with a significant effect varied in sensory intensity was roughly of the same size (4.42-5.34), whereas the spread in sensory intensities was notably larger (5.83-7.71) in analysis 2-4 (Table 3).
### Table 3. The spread in sensory intensities for attributes with significant sample effect ($p \leq 0.05$).

<table>
<thead>
<tr>
<th>Descriptor</th>
<th>Analysis 1</th>
<th>Analysis 2</th>
<th>Analysis 3</th>
<th>Analysis 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field experiment 1</td>
<td>Field experiment 2</td>
<td>---Field experiment 3---</td>
<td>---Field experiment 4---</td>
<td></td>
</tr>
<tr>
<td><strong>2010</strong></td>
<td><strong>2011</strong></td>
<td><strong>2011</strong></td>
<td><strong>2011</strong></td>
<td></td>
</tr>
<tr>
<td>8 Cultivars</td>
<td>1 Cultivar, 3 N Levels, 2 S levels</td>
<td>3 Cultivars, 2 N levels</td>
<td>2 Cultivars, 4 N levels</td>
<td></td>
</tr>
</tbody>
</table>

| Taste | Sweetness | 5.6-10.6 | 4.5-12.2 | 3.0-9.5 | 3.4-10.2 |
| | Bitterness | 5.5-10.9 | 2.8-10.3 | 5.2-12.7 | |
| Sensation | Pungency (nose) | 5.5-10.0 | 5.0-10.8 | | |
| | Pungency (mouth) | 5.1-9.6 | | | |
| | Astringency | 6.5-11.4 | | | |
| Texture | Juicyness | 5.8-10.7 | | | |
| | Crispiness | | | | |

On the basis of these results it is not possible to identify the analysis that resulted in the largest difference in sensory properties. Instead, it can be concluded that cultivar selection and changes in fertilization approach are both appropriate strategies if the aim is to produce kale with large differences in sensory properties. The fact that samples could only be significantly differentiated in terms of sweetness in analysis 1 is in excellent agreement with the results from the analysis of simple sugars where a significant effect was exclusively identified in analysis 1.

**Relationship between chemical measurements and sensory properties.** The assessment of results in the previous section proved that different strategies can lead to large variations in phytochemical content and sensory properties in the sample material. These differences in sample material permit a more thorough study on how the content of phytochemicals and sugars in kale is reflected in the sensory properties. In order to better understand the link between results from the chemical analyses and the sensory profile, multivariate data analyses in the form of PLS were applied. Only sensory attributes related to taste and mouthfeel were included in the analysis, since these are the sensory properties known to be related to the GLS, total phenolics and simple sugar contents and the analysis only encompassed variables that were found to significantly differentiate the samples. Scores and loadings plots from the PLS regression are shown in Figure 1a and 1b (analysis 1), Figure 1c and 1d (analysis 2), Figure 1e and 1f (analysis 3) and Figure 1g and 1h (analysis 4) and statistics for the four different PLS models is given in Table 4.
Figure 1. Continues on the next page.
Figure 1. Partial least squares (PLS) regression scores and loading plots for analysis 1 (a and b), 2 (c and d), 3 (e and f) and 4 (g and h). The plots only include variables with a significant product effect ($p \leq 0.05$). Data from the chemical analyses constitute X-variables (○) and sensory data constitute Y-variable (●).
Table 4. Variable influence on projection (VIP) summarizing the importance of the X-variables, both for X-model (chemical measurements) and Y-model (sensory profiling) for analysis 1-4 together with cross validated standard errors (cvSE). Only variables with significant sample effects were included in the partial least squares regression models.

<table>
<thead>
<tr>
<th>Chemical compounds</th>
<th>Analysis 1</th>
<th>Analysis 2</th>
<th>Analysis 3</th>
<th>Analysis 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Field</td>
<td>Field</td>
<td>---Field experiment 3---</td>
<td>2011</td>
</tr>
<tr>
<td></td>
<td>experiment 1</td>
<td>experiment 2</td>
<td>2011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VIP</td>
<td>cvSE</td>
<td>VIP</td>
<td>cvSE</td>
</tr>
<tr>
<td>Glucoiberin</td>
<td>0.70</td>
<td>0.36</td>
<td>1.02</td>
<td>0.81</td>
</tr>
<tr>
<td>Progoitrin</td>
<td>0.63</td>
<td>0.53</td>
<td>0.59</td>
<td>0.48</td>
</tr>
<tr>
<td>Glucoraphanin</td>
<td>0.71</td>
<td>0.81</td>
<td>0.96</td>
<td>0.61</td>
</tr>
<tr>
<td>Sinigrin</td>
<td>1.14</td>
<td>0.79</td>
<td>1.06</td>
<td>0.19</td>
</tr>
<tr>
<td>Gluconapin 4-hydroxy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucobrassicin</td>
<td>0.71</td>
<td>0.61</td>
<td>1.08</td>
<td>0.46</td>
</tr>
<tr>
<td>Glucobrassicin</td>
<td>0.90</td>
<td>1.10</td>
<td>1.66</td>
<td>0.51</td>
</tr>
<tr>
<td>4-methoxy glucobrassicin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gluconasturtin</td>
<td>1.12</td>
<td>0.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neoglucobrassicin</td>
<td>1.16</td>
<td>0.84</td>
<td>0.99</td>
<td>0.63</td>
</tr>
<tr>
<td>Total glucosinolates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.79</td>
<td>0.42</td>
<td>0.53</td>
<td>0.29</td>
</tr>
<tr>
<td>Fructose</td>
<td>1.10</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>1.14</td>
<td>0.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total sugar</td>
<td>1.23</td>
<td>0.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>1.27</td>
<td>0.78</td>
<td>0.85</td>
<td>0.33</td>
</tr>
<tr>
<td>Total phenolics</td>
<td>0.71</td>
<td>0.67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The scores and loadings plot from the PLS regression from analysis 1 (PC1 and PC2 describing 60% and 15% of the Y-variation, respectively) confirmed that the samples represented a large variation in the content of both GLSs and sugars (Figure 1a and 1b). TIARA, and to a lesser extent also LKH, LAVO and Reflex, was associated with a high intensity of bitterness, pungency (mouth) and astringency. The 4-methoxy glucobrassicin and glucobrassicin were the best predictors of these sensory properties. In previous studies, glucobrassicin (Drewnowski and Gomez-Carneros 2000), progoitrin (Pasini et al. 2011, van Doorn et al. 1998), sinigrin (Engel et al. 2002, van Doorn et al. 1998) and neoglucobrassicin (Engel et al. 2002) have been associated with bitterness in Brassica vegetables, but no study has previously found a relation between 4-methoxy glucobrassicin and bitterness, astringency and/or pungency (mouth). The HAT and HAST cultivars were perceived as having the sweetest taste. Glucose, sucrose, total sugar, DM, sinigrin and neoglucobrassicin were found to be good predictors of sweetness. The fact that sweetness was related to the content of simple sugars was expected. Sinigrin and neoglucobrassicin
are two bitter-tasting compounds (Engel et al. 2002, van Doorn et al. 1998) and the close association between these compounds and sweetness suggests that the content of these compounds was either too low for the panel to detect or that sugar masked the bitterness of these compounds. Sugar has previously been found to mask the bitterness of GLSs in *Brassica* vegetables (Beck et al. 2014, Zabaras et al. 2013). All the above-mentioned compounds, except glucobrassicin, were evaluated as being important variables (VIP values above 1) for the PLS regression model (Table 5).

### Table 5. Partial least squares (PLS) regression model statistics for analysis 1-4

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Experimental design</th>
<th>Year</th>
<th>Optimal number of principal components</th>
<th>R2X (cumulated)</th>
<th>R2Y (cumulated)</th>
<th>Q2 (cumulated)</th>
<th>RMSECV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8 Cultivars</td>
<td>2010</td>
<td>3</td>
<td>0.8</td>
<td>0.9</td>
<td>0.4</td>
<td>0.9-1.5</td>
</tr>
<tr>
<td>2</td>
<td>1 Cultivar, 3 N Levels, 2 S levels</td>
<td>2011</td>
<td>4</td>
<td>1.0</td>
<td>1.0</td>
<td>0.8</td>
<td>1.1-1.5</td>
</tr>
<tr>
<td>3</td>
<td>3 Cultivars, 2 N levels</td>
<td>2011</td>
<td>2</td>
<td>0.9</td>
<td>0.9</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>2 Cultivars, 4 N levels</td>
<td>2011</td>
<td>3</td>
<td>1.0</td>
<td>0.9</td>
<td>0.6</td>
<td>1.7</td>
</tr>
</tbody>
</table>

The scores and loadings plot from the PLS regression on results from analysis 2 show that samples were grouped along PC1 according to S supply (explaining 20% of the Y-variability) and along PC2 according to N supply (explaining 47% of the Y-variability) (Figure 1c and 1d). All GLSs included in the PLS regression were associated with samples fertilized with S and so were all the sensory attributes. A similar response to S fertilization was seen in a study on turnip roots (*Brassica rapa* ssp. *rapifera* L.) where the GLS content increased with enhanced S supply (Li et al. 2007). Except for glucobrassicin, which was present in larger amounts in samples fertilized at a high compared to a low N rate, the effect of N on the content of the GLSs included in the model was inconclusive (Figure 1c and 1d). The effect of glucobrassicin is explained by the results presented by Groenbaek and co-workers (2014) (Paper III), who found a higher content of glucobrassicin at the highest N level (185 kg ha⁻¹), and where the lowest (90 kg ha⁻¹) and highest (185 kg ha⁻¹) rather than the medium N level (135 kg ha⁻¹) resulted in a higher content of aliphatic GLSs (glucoiberin, progoitrin, glucoraphanin, sinigrin and gluconapin). The two indole GLSs, glucobrassicin and 4-hydroxy glucobrassicin, and the aliphatic GLS, sinigrin, were found to be the most important variables explaining the PLS regression model (Table 5).
The scores and loadings plot of the PLS regression from analysis 3 (PC1 and PC2 describing 70% and 21% of the Y-variation, respectively) showed a separation of samples by cultivar (Reflex, TIARA and HAT, in ascending order) and N level (90 and 230 kg ha\(^{-1}\), in ascending order) along PC2 (Figure 1e and 1f). In contrast, the PLS regression from analysis 4 (PC1 and PC2 describing 41% and 30% of the Y-variation, respectively) separated the samples by cultivar along PC1 (Figure 1g and 1h). A common conclusion drawn from these two models is that TIARA was perceived as being the most pungent (nose), whereas no general deductions could be made of the role of N level. TIARA was also perceived as the most bitter and pungent (mouth) in analysis 1 (Figure 1a and 1b). None of the common variables from the PLS regression models from analysis 3 and 4 obtained VIP values above 1 (Table 5).

Total phenolics content was not found to notably influence the PLS regression models (analysis 1 and analysis 4, Figure 1b and 1h) and was thus not a good predictor of any of the sensory characteristics (Figure 1b and 1h).

A comparison of PLS plots from analysis 1-4 shows no consistency between sensory properties and the GLS, total phenolics and/or simple sugar contents. This confirms that the relationship between phytochemical content and sensory properties cannot be ascribed to single factors but is a result of more complex interactions. This conclusion was also drawn by Zabaras and co-workers (2013). But in contrast to the present study they only studied fractionated extracts obtained from *Brassica* vegetables.

All the applied strategies (cultivar selection, fertilization approach and a combination of the two) succeeded in producing kale differing in sensory properties and in phytochemical and simple sugar contents. The largest effect on GLS, total phenolics and simple sugar contents was seen in the analysis that only included the cultivars grown in 2010 (analysis 1). Analysis 1 also provided the largest number of sensory attributes that could be used to significantly differentiate between samples, but the spread in the sensory intensities of the significant attributes was largest in analysis 2-4. Sulfur fertilization resulted in a higher concentration of GLSs and a higher intensity of bitterness, astringency and pungency (mouth). No distinct relationship could be established between individual or groups of GLSs or total phenolics across the four analyses. A strong connection was found between the content of simple sugars and sweetness, and sweetness was negatively correlated to bitterness, astringency and pungency. The TIARA cultivar was the most bitter and pungent (nose and mouth), whereas HAT and HAST were the sweetest. The results from the present study indicate that there is potential in producing kale with a high phytochemical
content without changing the sensory properties, and that both cultivar selection and changes in fertilization approach are appropriate strategies for modulating the phytochemical content and sensory properties.

**Abbreviations**

BONA, Halvhøj Kruset Bona; DM, Dry matter; FC, Folin Ciocalteu; FW, Fresh Weight; GA, Gallic acid equivalents; GLS, Glucosinolate; HAST, Høj Amager Sundby Torve; HAT, Høj Amager Toftø; HKK, Halvhøj Kruset Konserva; N, Nitrogen; LAVO, Lav Opretvoksende; LKH, Lav Kruset Harvester; S, Sulfur; TIARA, Halvhøj Ekstra Moskruset Tiara.

**Acknowledgement**

We would like to thank senior lab technician Birgitte Foged for preparation of vegetables and chemical analyses of samples together with Astrid Bergmann, Knud Erik Pedersen and Jens Barfod who were responsible for all field experiments.
Reference list


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We would like to express our gratitude to The Danish Council for Strategic Research for financial support.
Table of content graphics
5.1 Paper IV
Chapter 6

6.1 Discussion

The detailed discussions regarding the separate experiments have been treated within each paper (Chapter 2-5). In the following sections, discussions based on the overall hypotheses are provided as well as perspectives for the production of cabbage and kale by growers.

6.1.1 Hypothesis A.

A. The splitting of N fertilizer application to white cabbage and curly kale results in optimized N availability throughout the cultivation period which increases GLS but lowers flavonoid glycoside concentrations as compared to one dose or reduced N fertilization.

Splitting the N fertilizer resulted in increased concentrations of indole and total GLSs but reduced aliphatic GLS concentration compared to non-split fertilization in white cabbage plants (Paper I). Aliphatic GLS concentration in field-grown curly kale likewise decreased under N split fertilization (3*33% N) compared to one dose (100% N) and 2*50% N (Paper II). Similarly, decreases in aliphatic GLS concentrations have previously been found as a result of enhanced N fertilization (Schonhof et al. 2007, Stavridou et al. 2012). This was further confirmed by the results from Paper I (non-split dose), Paper II and Paper III (F1 hybrid Reflex), where elevated N application reduced the aliphatic GLS concentrations. An even availability of N throughout plant growth period in contrast to decreasing access would possibly favor secondary metabolism of GLSs as N will be more than sufficient for primary growth, as suggested by the C/N hypothesis (Bryant et al. 1983). Furthermore, indole GLSs were suggested to have a higher N requirement due to the N atom in the indole structure (Mithen et al. 2000), which is not found in the aliphatic GLS molecule. This was confirmed by increased indole concentrations under enhanced N fertilization (Paper I, Paper II).

The increase of indole and total GLS concentrations under split N dose fertilization in the greenhouse trial was not assigned to N alone, as this increase depended on S fertilization as well. Further differences are ascribed to the diverse experimental setups. Firstly the N application timing differed, as the non-split dose in the greenhouse experiment was
delayed compared to the field trial. Secondly the plant material differed with regard to variety, which has previously shown to have strong effects on GLS concentration (Cartea et al. 2008, Kushad et al. 1999). Thirdly, the different experimental environments might have caused the diversity in results. Natural conditions of biogeochemistry of the soil, light spectrum and proper development of the root zone are absent under greenhouse conditions, which on the other hand enable suspension of interfering factors such as flooding or heterogeneous soil conditions.

Split N dose fertilization (3*33% N) effects on flavonoid glycosides revealed increased kaempferol glycoside concentration when compared to one dose (100% N) and 2*50% N (Paper II). Besides that, the effect of split N dose amounted to differences in the 8 week old plants possibly due to the different levels of N applied. This might have been due to inhibitory N effects with respect to the flavonoid biosynthetic pathway rather than a balance between primary and secondary metabolism. A study on Labisia pumila revealed that enhanced N application resulted in reduced PAL activity, lower total flavonoid concentration and a positive relationship between the PAL activity and total flavonoid concentration (Ibrahim et al. 2011). Furthermore, quercetin and kaempferol flavonoid concentrations increased in response to decreased N levels in broccoli and tronchuda cabbage (Jones et al. 2007, Sousa et al. 2008). The same increase in quercetin and total flavonoid glycoside concentrations were found in Paper III for the F1 hybrid Reflex as a response to decreased N application. Therefore constant N availability (split dose) throughout the cultivation period was hypothesized as inhibiting flavonoid biosynthesis and lowering the concentration. This was rejected. The effect of split dose and high/low N fertilization was diminished throughout the cultivation period, as N treatment only had an effect on kaempferol concentration in 17 week old plants (Paper III).

The potential benefit of using split dose fertilization for vegetable producers is to optimize nutrient use efficiency, and for environmental purposes, such as reducing the risk of N leaching (Kitchen and Goulding 2001). A further argument for using this strategy could be the altered phytochemical composition. Different qualities in relation to health benefit have been ascribed to the individual GLSs and their breakdown products as well as a suggestion of down-regulation of the indole GLS neoglucobrassicin due to mutagenic effects (Barrett et al. 1998, Björkman et al. 2011, Hwang and Lee 2006, Jiang et al. 2013, Latte et al. 2011, Marconett et al. 2012). Development of fertilizer programs favoring the aliphatic GLS over the indole GLS biosynthesis might therefore be of interest. On the other hand, studies so far have concluded that an average consumer does not eat GLSs in
amounts sufficient to cause damage (Björkman et al. 2011, Latte et al. 2011, Steinbrecher and Linseisen 2009). With this in mind, split N dose fertilization to white cabbage plants is recommended, as total GLS concentration was highest under this treatment (Paper I). Regarding the kale, plants should be grown under low N fertilization and harvested later than the age of 17 weeks to reach the highest GLS concentration (Paper II). However, under low N levels, yields are decreased, which may contradict the production goals.

Flavonoid health beneficial effects have also been documented as they were shown to prevent development of cancer, asthma and possess anti-inflammatory and antioxidant activity (Cartea et al. 2011, Kim et al. 2006, Knekt et al. 2002, Zietz et al. 2010). For optimum flavonoid glycoside concentrations, kale plants should be harvested around the age of 13 weeks, as plant ontogeny presents an earlier optimum in flavonoid glycoside concentrations compared to GLSs. Furthermore, reduced N level should be applied to reach the highest concentrations of the afore-mentioned phytochemicals.

6.1.2 Hypothesis B.

B. Sulfur interacts with N in relation to GLS concentration when N is applied in split doses or increasing levels to white cabbage and curly kale.

Interactions between N and S fertilizers were found in relation to GLS concentration in white cabbage plants grown under greenhouse conditions (Paper I). In contrast, fewer interactions were seen in the field-grown curly kale (Paper III).

As a starting point we were interested in digging deeper into the interplay between N and S in relation to GLS concentration. Relationships between N and S fertilizers in correlation to GLS concentration have been investigated before, but not with N fertilizers applied in split dose, and there are only a few studies done under field conditions. There has been a natural interest in S fertilization in relation to GLS concentration, as S is incorporated into the GLS molecule as well as the amino acid precursor methionine (Fahey et al. 2001). The N/S ratio of the plant has likewise attracted attention due both to the balance between primary and secondary metabolism (Bryant et al. 1983, Mugford et al. 2011) and to the interactions between N and S fertilization in relation to plant N and S content besides GLS concentration (Brunold 1993, Omirou et al. 2009, Schonhof et al. 2007).

Our results confirmed a species specific relationship between the N/S ratio and GLS concentration for white cabbage (Paper I), supported by previous studies which tested
different *Brassica* species and varieties (Falovo et al. 2011, Omirou et al. 2009, Schonhof et al. 2007). Thus, the N/S ratio can be used to identify the optimum of GLS concentration in a given *Brassica*; however, it does show a degree of weakness. The levels of S applied have to be within certain parameters in order to create a wide enough range of both N/S ratio and GLS concentration so as to deduce the optimal N/S ratio. This was confirmed by a simple linear regression of the N/S ratio and GLS concentration in the two curly kale experiments reported in Paper III. The availability of just 30 kg S ha\(^{-1}\) in Experiment I in comparison with either 4 or 30 kg S ha\(^{-1}\) available in Experiment II resulted in negative relationships between aliphatic, indole and total GLS concentration and the N/S ratio in Experiment II, but not I (data not included). In addition, the diverse N responses caused by the genetic background of the cultivars selected might have had an influence (Paper III).

Therefore, optimal N/S ratio determinations may have to be done on a cultivar level rather than variety or species level. The timing of N fertilizer application played a decisive role, as non-split dose of N did not result in a relationship between N/S ratio and GLS concentration when compared to a split dose application (Paper I). Furthermore, the interdependent relationship of N and S in the plant assimilation process might complicate the interpretation of results and the ability to draw unambiguous conclusions (Brunold 1993, Koprivova et al. 2000, Mugford et al. 2011). For example, fewer interactions between N and S levels were seen under natural conditions in the field in relation to GLS concentrations (Paper III).

When so much emphasis is put on finding the right balance between the N and S fertilization and plant concentrations it has a bearing on the balance between primary and secondary metabolism. Increases in amino acids and proteins during primary metabolism will dilute the GLS concentration as seen for the F1 hybrid Reflex cultivar of curly kale in Paper III. A high responsiveness to N fertilization with respect to increase in biomass resulted in concentration decreases of many GLSs.

If these investigations could be converted into a guiding ratio of the applied level of N and S to optimum GLS concentration, it would possibly be of interest to growers. Or maybe even better, development of a model taking into account not only fertilizer application of N and S levels, but also cultivar and soil properties i.a., and providing the grower with information on how to obtain optimum GLS concentration.
6.1.3 Hypothesis C.

C. Cultivar selection of curly kale is reflected in growth pattern and thereby phytochemical concentration due to diverse genetic background.

The cultivars investigated in Paper III showed variations in growth pattern and phytochemical concentration due to genetic variety and responsiveness to increasing N application.

Cultivar selection has previously shown promising results in the investigations of phytochemical diversity within *Brassica* varieties. Thus, although the cultivars are closely related, several studies have revealed both quantitative and qualitative differences in GLS, flavonoid and hydroxycinnamic acid derivative concentrations (Cartea et al. 2008, Schmidt et al. 2010, Vallejo et al. 2003). The genetic background is the main force behind these differences; however, studies on environmental and cultivar interactions have shown cultivar dependent responses to growing season, N and S fertilizers, temperature and global radiation in relation to GLS and flavonoid composition and concentrations (Cartea et al. 2008, Charron et al. 2005, Paper III, Schmidt et al. 2010, Vallejo et al. 2003). These studies point to the importance of these factors for the plant phytochemistry and ask for continuing investigations on environmental effects to exploit the potential of cultivar selection and fertilization strategy in the production of health beneficial *Brassica* crops. Furthermore, genetic investigations and mappings of quantitative trait loci (QTLs) are important if the cultivar differences should be included in future breeding programs aimed at health beneficial properties of *Brassica* (Hennig et al. 2014).

The relationship between phytochemical concentration and growth pattern relates to the discussion on the balance between primary and secondary metabolism. In Paper III this balance was reflected in the different growth patterns revealed due to different N levels and the relationship was clarified better than would have been the case with an experimental setup with just one level of N. There was a good correspondence between the growth pattern, N responsiveness and GLS and flavonoid glycoside concentration of the different cultivars.

The Nordic Genetic Resource Center (NordGen 2014) preserves and maintains the genetic diversity among the old traditional cultivars which were cultivated earlier in the Nordic countries. This means there is a possibility of re-introducing cultivars and genes carrying beneficial traits which might have been bred out. The traditional cultivars of curly kale were investigated in this PhD project and relate well to an increasing interest among
consumers for the origin of the food. Furthermore, the introduction of the concept *New Nordic Food* by a group of Danish chefs in 2004 has increased people’s awareness of vegetables traditionally grown in The Nordic countries (Meyer et al. 2010). Urban farming, food communities in the cities (KBHFF 2014) and a growing interest in locally produced food have correspondingly strengthened the market for different cultivars. For example, the visual differences of white or purple carrots, red and white-striped beetroots and purple curly kale. This points to a desire and susceptibility among consumers to exploit genetic diversity, as well as a possibility for growers and retailers to broaden the selection of vegetables. Furthermore, the vegetable cultivar can be seen as a part of a produce’s *terroir*, which is further defined and influenced by factors such as climate, type of soil and topography (Kjeldsen et al. 2014). This term has gained increased attention among producers due to potential enhanced market value. However, knowledge of and familiarity with the characteristic labeling given to the products according to European Commission procedures are scarce among consumers.

6.1.4 Hypothesis D.

D. Nitrogen and S levels, split N dose, cultivar selection and frost exposure affect the phytochemical concentration and composition and thereby the sensory properties of curly kale.

The effects of N, S and cultivar selection on phytochemical concentration have been discussed in the previous sections. I will focus therefore on discussing frost exposure effects on phytochemicals and sensory properties in relation to all the cultivation strategies used.

The widely held belief in improved sensory quality of kale after exposure to frost, combined with further interest in split N dose fertilization were the main questions and driving forces behind the experiment reported in Paper II. The frost treatment did not reveal any differences in GLS or flavonoid glycoside concentrations, whereas sucrose and fructose concentrations changed slightly. Increasing concentrations of total sugars from August to September harvests might have been induced due to shorter photoperiod and lower temperatures above 0 °C as a cold acclimation in the plants (Kalberer et al. 2006, Theocharis et al. 2012). Aliphatic and total GLS concentration as well as kaempferol, quercetin and total flavonoid glycoside concentration within certain N treatments were also increased during this period. This could be seen as a cold acclimation as well, since
GLSs and flavonoid glycosides could potentially bind free water due to both glycosides and a high number of hydroxyl groups (Swiderski et al. 2004). However, these changes were mostly ascribed to ontogenetic effects.

Phytochemical variation throughout the cultivation period enables the growers to choose an output with a certain phytochemical composition. For example relatively small plants would have lower GLS concentrations compared to plants harvested later in the season, and flavonoid glycoside concentration should peak in approximately 13 week old plants, according to our results (Paper II). This adds further potential in terms of the production of baby leaves for the market, as is already the case for both green and purple kale cultivars versus the full grown kale leaves normally used in the traditional Danish dishes.

An experiment with whole plants exposed to frost would be interesting for future research. Most likely the separation of leaves from the main plant and the roots affected the plant physiology and response to frost exposure in our study. Likewise the moving of the plant material from a natural environment into plastic bags has had an impact on the plant. The challenge in the experimental setup consisted in ensuring the provision of samples with and without frost treatment simultaneously, as the sensory panel cannot evaluate samples days or weeks apart. Controlled frost exposure is more reliable than counting on degrees below 0 °C in the field at a certain time, including with respect to obtaining homogenous samples and for planning panel attendance. However, conditions closer to the natural e.g. climate chamber with room for full size plants grown under temperature and day length conditions resembling those outdoors during cold acclimation period would probably have led to different results.

The stability of the chemical composition during frost exposure was reflected in the sensory profile, where the most significant effect of frost related to an alteration in texture (Paper II). Bitterness, astringency or pungent aroma and flavor, which usually are barriers to cabbage consumption (Drewnowski and Gomez-Carneros 2000), were not affected by frost exposure. Curly kale contains a high level of fibers (6.2 g 100 g⁻¹ fresh weight (Danish Food Composition Databank 2014)) and is considered a coarse vegetable. Served raw, the texture of kale might contribute to the reduced liking and acceptance of cabbages in general. The changes in texture due to frost in our study amounted to less crispness after frost exposure (Paper II). This resembles a softer and less coarse product, which is likely seen as a positive sensory property for the consumer. Another reason for the assumed better quality of kale having been exposed to frost could be due to the reduced number of insects such as aphids or cabbage flies.
The cultivars used in the MaxVeg project were a matter of availability from NordGen (NordGen 2014). In the first year of the project, seven traditional cultivars and one F1 hybrid were grown under standard conditions and screened based on sensory properties by a sensory panel (Paper IV). From these screenings, the three cultivars tested in Paper III were chosen based on responsiveness to N supply and their sensory scores, particularly sweetness, bitterness, astringency, and pungency. The overall project hypothesis suggested that stronger and bitterer tasting root vegetables and cabbages would have a higher concentration of health beneficial phytochemicals than the mild and sweet ones. And traditional cultivars would be bitterer due to less breeding towards sweeter produce. This hypothesis was rejected, as some old cultivars turned out sweeter and milder than others and the F1 hybrid was actually among the more bitter cultivars (Paper IV). Cultivar selection and fertilizer strategy thereby resulted in kales possessing different sensory properties. Fertilizer strategy effects were mostly ascribed to S application as GLS concentration increased when S was applied and the panel detected higher intensities of bitterness, astringency and pungency (mouth) (Paper III, Paper IV). However, no distinct relationship between the levels of GLSs normally considered as bitter or astringent and the actual sensory scores of these attributes was found (Paper IV). In the pre-trials low N application seemed to increase the perceived sweetness as similarly suggested for pak choi (Talavera-Bianchi et al. 2010), but this was not unambiguously confirmed in the later investigations (Paper II, Paper IV). Furthermore, high responsiveness to N application for the GLS concentrations in F1 hybrid Reflex compared to the traditional cultivars Tiara and HAT (Paper III) was not reflected in uniform N effects on the sensory properties (Paper IV). As sugar content was a good predictor for perceived sweetness of the kale cultivars (Paper IV), this seemed a good tool for cultivar selection with the aim of producing kales which will meet with increased consumer liking. Sweetness is known to increase vegetable liking and has been found to have a masking effect on bitter GLSs (Beck et al. 2014, Cox et al. 2012). This reflects a complex interplay between sugar and phytochemical content as well as sensory properties, but also that there is a possibility of producing a vegetable with possibly increased health value without changing its sensory properties (Paper IV). Further cultivar selection might possibly contribute to kale production targeted at consumers with specific interests or taste preferences such as chefs and restaurants.
Chapter 7

7.1 Conclusions and Perspectives

Throughout this PhD project many thoughts and plans have been discussed with the overall aim and hypotheses as starting points. A lot of them turned out to be exactly those experiments and discussions reported in this thesis, whereas others were discarded along the way. The conclusions from this PhD project and some perspectives for future research are presented in what follows.

Split dose fertilization of N to white cabbage and curly kale was investigated in relation to GLS and flavonoid glycoside concentrations and revealed significant changes. This relationship was studied for the first time during this PhD project and should provide a further motive for both scientists and growers to pursue this method for purposes other than increased yield and reduced risk of N leaching. Further investigations, including more Brassica varieties and cultivars, are suggested, as this study revealed diverse genetic background as having significant impact on the degree of response to N application. This concerned yield, growth pattern and phytochemical concentration and composition. The conclusion is therefore that there was a close relationship between the primary and secondary metabolism in relation to biosynthesis of phytochemicals, even though our results did not conform strictly to the carbon/nutrient hypothesis. Moreover, studies with a wider range in the N levels applied might possibly add to existing knowledge of the diverse responses, as at least one cultivar seemed to be susceptible to more N with respect to yield increase. Knowledge about the cultivar differences provides a basis for refined fertilizer management, as well as possibilities for choosing cultivars with specific health beneficial or agronomic traits. It points to the potential benefits of re-introducing traditional cultivars and including more agronomic traits and possibly S fertilization as a factor in determining the relationship to health beneficial cultivar qualities of cabbage and kale.

Increased S application enhanced GLS concentration in both white cabbage and curly kale plants. Furthermore, S and N fertilization interacted to a higher degree with respect to GLS concentration under greenhouse conditions as opposed to field conditions. This conclusion underlines the necessity of S fertilization in relation to GLS concentration. Furthermore, the results reflect the challenges of transferring methods for GLS modulation developed under greenhouse conditions to the field. As both N and S are incorporated into primary and secondary metabolites, studies with isotope labeling would help clarify how the atoms
are distributed dependent on availability and possibly the N/S ratio of the applied fertilizers as well as in the plant material.

Frost exposure did not change phytochemical concentration and composition, whereas during the cultivation period up to 4-fold increases in aliphatic and total GLS concentrations were revealed. Whether the climatic conditions had an influence on these increases cannot be ruled out. Therefore, future studies on kale grown under stable climatic conditions, such as unchanged temperature and light conditions, should clarify this. Equally, an experiment where plants of the same age are exposed to different lower temperatures would be of interest. In view of the relatively big changes in GLS concentration throughout plant growth period, a study on sensory properties of plants at different stages with respect to ontogeny and cold acclimation is suggested.

Cultivar selection and N and S fertilization approaches resulted in kales with different sensory profiles. The concentration of phytochemicals could not predict the perception of bitterness, astringency and pungency, but sugar content increased the sweet perception of the kale. There is therefore a possibility of producing kales with increased health value without changing their sensory properties. Whether these differences detected by the sensory panel could also be observed by an average consumer would be another interesting path to follow. The increased liking of vegetables due to sweetness points to more research within the area of altering sugar content in combination with phytochemical concentrations. Similarly, analyses of a broader spectrum of phytochemicals, such as carotenoids and fibers, which might influence the sensory properties and the relation to sensory properties, could provide further evidence as to what is actually causing the diversity. Furthermore, many cultivars have not yet been investigated under different N fertilizer levels. With the diverse responsiveness of the cultivars to N fertilization and a relatively high number of sensory attributes which differed between cultivars, the implication is that a combination of these two cultivation strategies would be suitable for further investigations.
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